RESEARCH ARTICLE

# Ani-survivin DNAzymes Inhibit Cell Proliferation and Migration in Breast Cancer Cell Line MCF-7

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## Abstract

**Survivin, a new member of the inhibitor of apoptosis protein (IAP) family, both inhibits apoptosis and regulates the cell cycle. It is overexpressed in breast tumor tissues. In this study, we designed two survivin specific DNAzymes (DRz1 and DRz2) targeting survivin mRNA. The results showed that DRz1 could decrease the expression of survivin by nearly 60%. Furthermore, DRz1 significantly inhibited cell proliferation, induced apoptosis and inhibited migration in MCF-7 cells. In addition, down-regulation of survivin expression was associated with increased caspase-3 and -9 activities in MCF-7 cells after 24 h transfection. In our experiments, the efficacy of DRz1 to influence survivin levels and associated effects were better than DRz2. Survivin-DRz1 might have anti-tumorigenic activity and may potentially provide the basis for a novel therapeutic intervention in breast cancer treatment.**

**Keywords:** DNAzymes - proliferation - apoptosis - survivin - breast cancer

*Asian Pacific J Cancer Prev,* **13 (12)**, 6233-6237

## Introduction

Breast cancer remains one of the top threats to the health of women (Baxi et al., 2012), the second leading cause of cancer-related deaths among women in China. Survivin is usually found at low level in normal cells but it is highly expressed in most tumors (Xu et al., 2012). High survivin expressions in breast cancer cells correlated to poor prognosis, decreased apoptosis, and increased resistance to drugs. As a functional protein, survivin, like other members of inhibitor of apoptosis proteins, suppresses apoptosis by binding to caspase-3, 7 and 9 (Barnes et al., 2006). Besides that, previous reports suggested that survivin played major roles in cell division where it was dominantly induced during G2/M phase to assist mitosis and cytokinesis (Gritsko et al., 2006). Therefore, it may be an attractive target for antitumor gene therapy.

Deoxyribozymes (DNAzymes or DRzs) are synthetic, single-stranded catalytic DNAs which can be engineered to bind by Watson–Crick base pairing to complementary sequences in a target messenger RNA (mRNA) and cleave it at predetermined phosphodiester linkages (Mosmann, 1983). The 10-23 model has been proposed as a general model for DNAzymes. A 10-23 DNAzyme has a catalytic domain of 15 deoxyribonucleotides, flanked by two arms, each with a 7-9 deoxyribonucleotide substrate-recognition domain. In vitro analysis has shown that this type of DNAzyme can cleave substrate RNA effectively at purine and pyrimidine junctions (Zhou et al., 2009). Furthermore, 10-23 DRzs have been shown in both in vitro and in vivo to inhibit the expression of their target and dependent genes. Moreover, DRzs are able to attenuate the progression of a variety of pathologies in animal models and show significant potential as therapeutic tools. The properties, functionalities and diverse forms of DNAzymes were recently reviewed by McManus (McManus, 2010) and Schlosser (Schlosser, 2009).

In the present study, we designed 10-23 DNAzymes against human survivin mRNA to investigate its role in MCF-7 cell line. It is could be a potential as tools for breast cancer gene therapy by determining their effects on cell proliferation and apoptosis.

## Materials and Methods

*DNAzyme design*

Two DNAzymes, designated DRz1 and DRz2, were designed as described before (Wang et al., 2011) via methodology outlined by Dass et al. (2008). Briefly, a region of 10-23 DRz was made by incorporating 8 bps arms complementary to the target gene at each side of the catalytic motif (Verma et al., 2010). As a control, an inactive DNAzyme was designed based on the flip sequence of DRz1. Both DNAzymes were synthesized by Shanghai Sangon Biotechnology Co. Ltd (Shanghai, China). The sequences of human survivin mRNA templates and corresponding DNAzymes were designed as follows (arrows indicate the cleavage point):

Template-1: 5'-CGGAGCG'UGGCCGAGGC-3'
DNAzyme 1 (DRz1): 5' GCCTCGGTCCGCTCCG 3'
Template-2: 5' -GGAGCCAGA'UGACGACCCC- 3'

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DNAzyme 2 (DRz2):5'-GGGGTCGTCTCTGGCTCC-3'
Inactive DNAzyme: 5'-GCCTCGCCTGGCTCCG- 3'

*Cell lines and cell culture*

The human breast cancer cell line MCF-7 used in this study and purchased from the Cell Bank and Type Culture Collection of the Chinese Academy of Sciences (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China). Cells were maintained and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco/ Life Technologies, Grand Island, NY, USA), 100 IU/ ml penicillin, 100 IU/ml streptomycin and 2 mmol/l L-glutamine in a humidified atmosphere (5% $CO_2$ , 37°C). Cells were counted in suspensions using a Cedex analyzer (Innovatis AG, Bielefeld, Germany).

*Transfection*

Transfection was performed according to the manufacturer's protocol with Lipofectamine 2000 (Invitrogen/Life Technologies, Grand Island, NY, USA) and 10 μl expression vector in 250 $\mu$l serum-free medium. The concentration of the DNAzymes used was 1.0 μg in 250 $\mu$l medium (4 $\mu$g/ml). After 6 h, the medium was replaced with fresh serum-containing DMEM and incubated for an additional 24 h.

*Cell viability assay*

Cell proliferation and concomitantly DRz cytotoxicity was assessed via the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which is based on the reduction of a yellow soluble tetrazole to an insoluble purple formazan in respiring cells (Zhou et al., 2009). Cells were seeded at an initial density of $5\times10^4$ cells/well in a 96-well plate and transfected with 0.4 $\mu$g DRzs in 100 $\mu$l medium (4 $\mu$g/ml) for 24, 48 and 72 h. At the end of the treatment, media containing the DRzs were carefully aspirated and 200 μl medium with 20 μl of a 5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, USA) solution in PBS was added to each well. After 4 h incubation at 37 °C, the medium was removed and 100 μl DMSO was added to each well. The optical absorbance (A) was measured at 490 nm using a BioTek ELX800 multiwell reader (BioTek, Winooski, VT, USA). The percentage of viable cells was calculated according to: CV (%) = (A of experimental group/A of control group) ×100%.

*Real time-PCR*

Human survivin and β-actin (housekeeping control) PCR primers were designed using Primer Express software (Perkin-Elmer Biosystems, USA) based on their published sequences: survivin (381 bp): 5'-ACCGCATCTCTACATTCAAG -3' (forward) and 5'-CAAGTCTGGCTCGTTCTC-3' (reverse); β-actin (318 bp): 5'-ATCATGTTTG AGACCTTCAACA-3' (forward) and 5'-CATC TCTTGCTCGAAGTCCA-3' (reverse). Total RNA of MCF-7 cell was isolated with TRIzol reagent (Invitrogen/Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. To avoid DNA contamination, total RNA was treated with RNase-free DNase I (Takara, Kyoto, Japan) for 60 min at 37 °C and extracted once again with the TRIzol reagent. The RNA purity was determined from the ratio of 260/280 nm absorbance and the RNA integrity was assessed by determining the intensity of the 28S and 18S rRNA bands after formaldehyde agarose gel electrophoresis. Total RNA (2 $\mu$g) was subjected to reverse transcription using a RevertAidTM First-Strand cDNA Synthesis Kit (Fermentas Inc., Glen Burnie, MD, USA) with a random hexamer primer and 2 $\mu$l cDNA solution was used for real-time PCR. Genes were amplified in a 25 μl reaction volume using SYBR Green (Applied Biosystems, Forster City, CA, USA) on a MiniOpticon™ Real-time PCR System (Bio-Rad, Hercules, CA, USA). The temperature profile comprised an initial denaturation step at 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s, 58°C for 15 s and 72 °C for 10 s before melting curve analysis. The specificity of the amplified products was evaluated via agarose gel electrophoresis and was further verified with automated cycle sequencing. To ensure consistency in threshold cycle (Ct) values, duplicate reactions were performed and mean Ct values were used for calculating the relative expression levels. The Ct values were analyzed as described previously by Zhou et al. (2009) and the normalized Ct values for each gene were subjected to a Student's t-test with two-tailed distribution to determine statistical significance (95% confidence interval). Reactions were carried out in triplicate and the mean value was used. For standardization, β-actin was used as an internal control of each sample.

*Western blotting analysis*

After transfection, cells were washed twice with pre-cooled PBS and subsequently, 106 cells were treated with RIPA buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate) containing protease inhibitors (1% cocktail and 1 mM PMSF). Total proteins were separated on 15% SDS-PAGE and transferred to PVDF membranes. The membrane was blocked with Tris-buffered saline with 0.1% Tween 20 (pH 7.6, [TBST]) for 1 h at room temperature and the PVDF membrane was immunoblotted overnight at 4 °C with the first antibody (see below) solution (1:1000). After washing twice with TBST, the membrane was incubated with HRP-labeled secondary goat anti-mouse IgG2a-B antibody (sc-2073) for 1 h at room temperature and washed three times with TBST. The final detection was performed with enhanced chemiluminescence (ECL) western blotting reagents (GE Healthcare, Piscataway, NJ, USA) and membranes were exposed to Lumi-Film Chemiluminescent Detection Film (Roche Applied Science, Rotkreuz, Schwitzerland). Loading differences were normalized using a monoclonal β-actin antibody. The primary antibodies used in this study included anti-survivin (SC-65610), anti-caspase-3 (SC-7272), anti-caspase-9 (SC-70507), and β-actin (SC-130301) and were all acquired from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

*Flow cytometry with Annexin V/Propidium iodide staining*

For quantitative assessment of apoptosis, cells were double-stained with Annexin V-FITC and propidium iodide

(PI) and analyzed by flow cytometry on a FACSAria™ flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). Briefly, cells ($1\times10^5$) were harvested and stained with Annexin V-FITC and PI double staining kit (Kaiji Bio Co., Nanjing, China) according to the manufacturer's instructions. Cells were immediately analyzed by flow cytometry and the signals from apoptotic cells were localized in the lower right quadrant of the resulting dot-plot graph.

*Caspase-3 and caspase-9 activation assays*

The caspase-3 and caspase-9 colorimetric assay kits (Kaiji Bio Co., Nanjing, China) were used to determine caspase-3 and caspase-9 activation in SMMC-7721 cells, according to the manufacturer's instructions. Briefly, cells were lysed by incubating with cell-lysis buffer on ice for 1 h, and the resulting mixture was centrifuged at 10,000 × g for 1 min. Enzymatic reactions were carried out in a 96-well microplate. To each reaction sample, 50 μl cell lysate was incubated with substrate for 4 h at 37 °C, before measurement of the absorbance at 405 nm with a BioTek ELX800 multiwell reader (BioTek, Winooski, VT, USA). Two additional controls, one without cell lysate and the other without substrate were included. Total protein was determined by the Coomassie brilliant blue method.

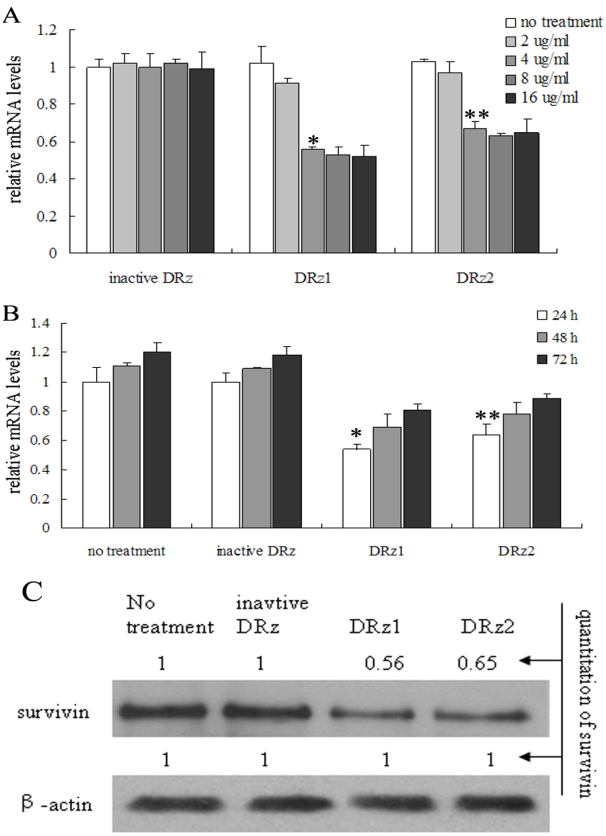


**Figure 1. Effect of DRzs on the Expression of Survivin in MCF-7 Cells.** (A) mRNA expression of survivin was significantly inhibited by different concentrations DRzs. The cells were transfected with the indicated concentrations DRz1, DRz2 or inactive DRz for 24 h. Data shown are means + SD of normalized relative survivin mRNA levels ($n \geq 3$). *denotes a significant difference to control ($P < 0.05$). (B) mRNA expression of survivin was significantly inhibited by DRzs. The cells received either no treatment or were transfected with 4 $\mu$g DRz1, DRz2 or inactive DRz after 24 h, 48 h and 72 h. Data shown are means + SD of relative IGF-IIP3 mRNA expressed compared to control (no treatment) ($n \geq 3$). *denotes a significant difference to control ($P < 0.05$). (C) Survivin protein expression was quantified via densitometry and compared with the "no treatment" control. The maximal grey value was set to 1 and all other results were expressed relative to this value (black) ($n \geq 3$)

*Wound healing assay*

To determine the effect of DRzs on migration of MCF-7 cells in vitro, cells were seeded at a density of 1×105 cells in 24 wells and grown to about 90% confluence after 24h. Medium was removed and cell mono-layers were wounded by manually scraping the cells with a 1 ml plastic pipette tip. Debris was removed from cultures by washing with PBS twice, and cells were then cultured in serum-free medium containing 0.4 μg DRzs and 2 μl Lipofect-amine 2000 for 6 h; after 6 h, the medium was replaced by serum-containing medium and incubated for an additional 24 and 48 h. Images were captured immediately after wounding and 24 h later, and wound closure was monitored with a UOP DSZ500X inverted microscope (UOP Microscope, Chongqing, China). Wound sizes were verified with image scales to ensure that all wounds were initially the same width. The migration distance and migration inhibitory rate were calculated using the following formula: migration distance= (wound width at the beginning–wound width after treatment)/2 (μm); migration inhibitory rate= (average migration distance in the control group–average migration distance in the treatment group) / average migration distance in the control group×100%.

*Statistics and data analysis*

Measurements were performed in triplicate and results are expressed as a means ± SD. Data were obtained from at least three independent experiments. Analysis of variance (ANOVA) for multiple comparisons was carried out using statistical analysis software (SPSS, Chicago, IL, USA). In all cases, values of P below 0.05 were considered to indicate significant differences.

## Results

*Inhibition of survivin mRNA and protein expression by DRzs*

We hypothesized that by preventing transcription of survivin mRNA via selective cleavage with suitable DRzs, IGF-II protein levels can be significantly reduced. Real time-PCR and western blotting analysis were used to detect the effects of DRzs on target gene expression in various concentrations and various times (Figure 1) to determine the optimal incubation concentration.

The results in Figure 1A show that both DRz1 and DRz2 were able to inhibit survivin mRNA expression. These effects were concentration dependent. Figsure 1B show a time-dependent response to the incubation with 4 $\mu$g/ml after 24 h, 48 h and 72 h of treatment with survivin-DRzs. However, these effects were transient, since both mRNA levels increased with time to reach near normal levels after 72 h of treatment. To evaluate the capacity of the DRzs to inhibit survivin protein expression in MCF-

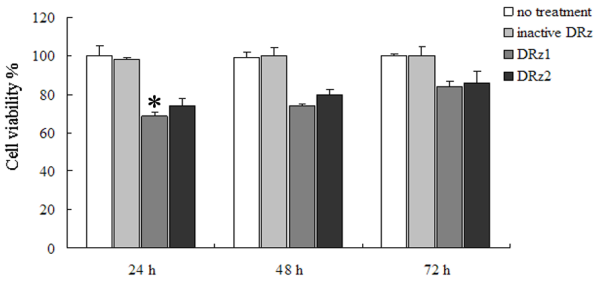


**Figure 2. Assessment of Cell Proliferation Via the MTT Assay.** Cell proliferation and survival of MCF-7 cells (initial density 5×104 cells/well) in the presence of 4$\mu$g/ml of the survivin-DRzs for maximally 72 h. All data are means ± SD from triplicates (n ≥ 3); * indicates a significant difference (P < 0.05)

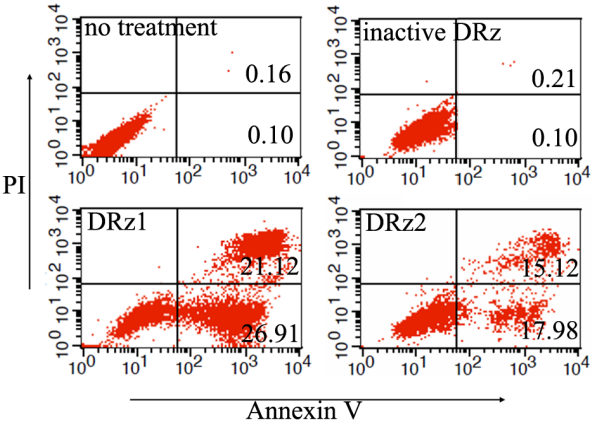


**Figure 3. Representative Flow Cytometric Assessment of Apoptosis Via Annexin V and Propidium Iodide (PI) Staining.** MCF-7 cells were treated with 4 $\mu$g/ml DRz1 and DRz2, and kept in culture for 24 h. Every 24 h, ~105 cells were analyzed with flow cytometry following Annexin-V-FITC/PI staining to evaluate changes in the apoptotic population

7 cells was treated with the DRzs for 24 h and survivin protein levels were evaluated on Western blot. As shown in Figure 1C, both DRz1 and DRz2 were generally able to inhibit survivin protein expression in MCF-7 cell.

*Effects of DRzs on cell proliferation*

The cytotoxicity of DRzs was determined from their effects on proliferation of human MCF-7 cells. Following 24 h, 48 h and 72 h treatment with DRzs, the MTT assay showed that DRzs could inhibit the growth MCF-7 cells (Figure 2). Compared to control, DRz1 inhibited MCF-7 cell proliferation by approximately 31.3 ± 5.1%, 26.4 ± 1.6% and 16.1 ± 1.3 after 24, 48 and 72 h treatment respectively. These results showed that the proliferation presents itself in the same transient fashion as the mRNA and protein levels (Figure 1).

*Flow cytometric evaluation of DRz- induced apoptosis in MCF-7 cell*

To further assess the capacity of the DRzs to induce apoptosis in MCF-7 cells, MCF-7 cells were treated with the DRzs and the externalization of phosphatidylserine via Annexin V-FITC staining was assessed by flow cytometry. The results in Figure 3 are representative for the performed experiments and show that both DRz1 and DRz2 maximally induced early stage apoptosis after 24 h, with 26.91 and 17.98% respectively, DRz1 more

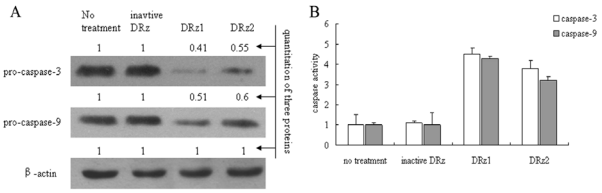


**Figure 4. (A) Western Blot Analysis. MCF-7 Cells were Transfected with 4 $\mu$g/ml DRz1 or DRz2.** Pro-capsase-3 and 9 levels were assessed after 24 h incubation via western blotting. A representative blot of three independent experiments is shown (n = 3). Densitometry analysis showed that the protein signal densities in DRz1 treated cells were lower than in control cells (no treatment). (B) Effect of survivin-DRzs on caspase-3 or 9 activities in MCF-7 cells. The cells were kept in culture, treated with survivin-DRzs for 24 h, and subsequently analyzed for caspase-3 or -9 activities (n ≥ 3)

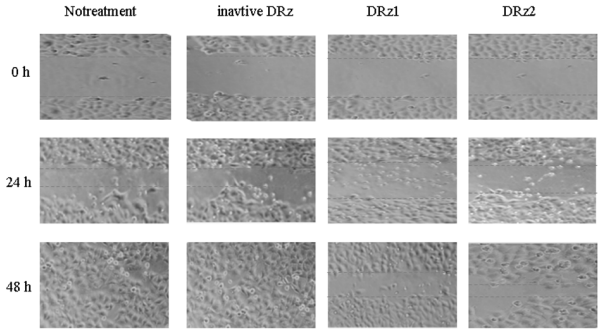


**Figure 5. The Effects of DRzs on the Migration of MCF-7 Cells.** DRzs inhibited migration of MCF-7 cells in vitro. Confluent MCF-7 cell monolayers were wounded by scraping and then treated with 4 μg DRz. Cell migration to the wound surface was monitored after 24 and 48 h. A representative image (magnification×100), bar = 200 μm

efficiently induces late stage apoptosis.

*DRz1 induces apoptosis through caspase activation in MCF-8 cells*

Caspase-3 is widely activated in all major apoptotic signal transduction pathways. We tested pro-caspase-3 and -9 expressions after 24h DRzs-treatment. In comparison to controls, pro-caspase-3 and pro-caspase-9 expression levels decreased significantly in DRz1-treated MCF-7 cells (Figure 4). To confirm that DRz1 and DRz 2 induce apoptosis via the caspase-dependent intrinsic pathway, we measured caspase-3 and 9 activities in response to 24 h of DRz treatment in MCF-7 cells. Compared to controls, the activities of caspase-3 and 9 increased significantly and in conformity of all previous experiments, DRz1 showed the strongest caspase induction (Figure 4B). These results suggested that DRz1 and 2 induce apoptosis in a caspase-dependent manner.

*Inhibition the migration of MCF-7 cells by DRzs*

To determine the effect of DRzs on migration of MCF-7 cells in vitro, the Wound healing assay was used. The results showed that DRzs could inhibit the migration of MCF-7 cells in vitro (Figure 5). After treatment with 4 μg DRz1 for 48 h, cells remained creviced, while untreated wounds healed completely. This inhibitory effect was time-dependent. Quantitative analysis showed that MCF-7 cells treated with 4 μg DRz1 for 24 h, 48 h reduced the

migration of MCF-7 cells by 14%, 60%, respectively. Our results are supportive of the idea that DRz1 inhibits the motility and migration of MCF-7cells.

## Discussion

Survivin, a new member of the inhibitor of apoptosis protein (IAP) family, both inhibits apoptosis and regulates the cell cycle (Yuan et al., 2012). It is overexpressed in breast tumor tissues, but hardly expressed in most normal tissues (Montazeri et al., 2012; Yakirevich et al., 2012). Therefore, it may be an attractive target for antitumor gene therapy.

In this study, utilizing the MCF-7 cell line, we demonstrated novel anticancer effects of survivin DRzs and suggested possible mechanisms underlying their anticancer effects in vitro. Here, we used DNAzyme technique to suppress the expression of survivin to explore its importance, role and functions. Under this condition, we observed how survivin participates in the proliferation, growth, and migration of breast cancer cells. DRz1 was shown to be more effective than DRz2 in down-regulating the expression of survivin, which is likely a direct result of the careful design of DRz1's action against the initiator of survivin mRNA (AUG). Both the reduction in mRNA and protein expression was in the same order of magnitude and was in the order of 50-60%. Cell proliferation was transiently attenuated by nearly 30% in MCF-7 cells after the first 24 h. Further evaluation over a large population of cells via flow cytometry showed that both DRzs could induce early apoptosis in 26.91% of the MCF-7 cell population. Initial assessments showed that DRz1 could significantly down-regulate the expression of pro-caspase-3 and 9, and concomitantly increase caspase-3 and caspase-9. These results suggest that DRz1 might induce apoptosis caspase-dependently through the intrinsic and mitochondrial pathway in MCF-7 cells. Similar apoptosis promoting effects were reported in Dz13, a DNAzyme targeted at c-jun that induces apoptosis in a caspase-2 dependent way. Therefore, survivin DRz1 may inhibit the migration of MCF-7 cells.

Overall, downregulation of the expression of survivin may induce apoptosis and inhibit migration of MCF-7 cells. In our results generally demonstrate that targeting survivn DRz1 is a promising and potential new therapeutic option in combating breast cancer.

## Acknowledgements

This work was partially supported by the Governmental Education Department of Jilin Province under grant # 2008087 (L. S. and Z. M.).

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