

# Intron-specific shRNA-mediated downregulation of survivin and promotion of apoptosis in HeLa cells

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Received January 20, 2016; Accepted June 2, 2017

DOI: 10.3892/ol.2017.6996

**Abstract.** Overexpression of the survivin gene contributes to tumorigenesis; it has been recognized as an important target for cancer therapy. In the present study, survivin expression was suppressed using recombinant plasmid mediated short hairpin RNAs (shRNAs) that were constructed to target exonic or intronic sequences of the survivin gene. In addition, a negative control shRNA was constructed. HeLa cells were transfected with specific shRNA constructs and the blocking efficiency of each shRNA was assessed at the mRNA and protein levels; and the five shRNA constructs with higher blocking efficiency were selected. Cell apoptosis was assessed by flow cytometry (FCM) following Annexin V-fluorescein isothiocyanate/propidium iodide double staining. Hoechst staining was used to detect the morphological diversity of the nuclei in apoptotic cells. The results demonstrated that survivin expression was effectively reduced by the transfection of shRNAs in HeLa cells. In addition, the apoptotic rates of the shRNA-treated groups were significantly increased compared with the negative control group according to the FCM results. The nuclei of HeLa cells exhibited apoptotic characteristics in the shRNA-treated groups as identified by Hoechst staining. Survivin-targeting shRNAs effectively downregulated the expression of the gene and markedly increased the apoptotic rate of HeLa cells. Data from the present study also indicated that the intron-specific shRNA demonstrate a high efficiency of inhibition of survivin expression and were able to induce cell apoptosis of HeLa cells through RNAi, potentially providing novel target sites for tumor therapy. In conclusion, the present study suggests that intron-specific blocking of survivin by RNAi may provide a tool for anticancer therapy.

## Introduction

The survivin gene is located at the 17q15 region chromosomal region and contains four exons and three introns (1). Survivin was firstly separated from a human genomic library using the complementary DNA (cDNA) of effector cell protease receptor-1 (EPR-1) through hybridization screening (2). Survivin, containing only one baculovirus inhibitor of apoptosis protein repeat (BIR) domain, is the smallest member of the mammalian inhibitors of the apoptosis protein (IAP) family (2,3). Survivin is detectable during fetal development and is highly expressed in various malignant tumors, but is undetectable in adult terminal differentiation tissues (2,4-6). Survivin serves multiple functions in cell division, apoptosis, metastasis and angiogenesis (7). It has been reported that survivin acts as an intermediary between cell apoptosis and cell cycle checkpoints, serving an important role in reducing cellular apoptosis and regulating mitosis (8). Survivin suppresses apoptosis through inhibition of the terminal effectors caspase-3 and caspase-7, promoting the differentiation of tumor cells (6,9). Survivin expression peaks at the G<sub>2</sub>/M phase of the cell cycle, where it associates with mitotic spindle microtubules; its overexpression is required to protect cells from apoptosis during mitosis (10). It has previously been reported that the overall survival rate of patients that express high levels of survivin is decreased, and that survivin is an indicator of poor prognosis (11,12); the recurrence rate of these patients was increased and patients were not sensitive to radiotherapy and chemotherapy (13). Therefore, survivin may be a promising target for anticancer therapy (14).

RNA interference (RNAi) is the sequence-specific silencing of genes induced by endogenous or exogenous 21-23 nt double-stranded RNA in cells (15). RNAi is a feasible and effective method of inhibiting the endogenous expression of target genes, and has been widely employed in the functional studies of genes *in vivo* and *in vitro* (16,17). Two modified strategies of RNAi technology have been used to block gene expression in mammalian cells, including small interfering RNA (siRNA) and vector-mediated short hairpin RNA (shRNA) (18). Compared with shRNA vectors, siRNAs are more readily synthesized and delivered into cells; however, siRNA-mediated gene silencing is transient and siRNAs are

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**Key words:** survivin, RNA interference, short hairpin RNA, apoptosis, intronic

susceptible to RNase degradation (19-21). shRNAs, on the other hand, can be used for long-term gene silencing and can also be propagated indefinitely (22,23). Once delivered into cells, shRNAs are cleaved into active siRNAs by Dicer, which then induce the homology-dependent degradation of cognate mRNA (24).

In the present study, the vector-derived shRNA technique was utilized and the recombinant plasmid pGP-U6-GFP-Survivin-shRNA was constructed, using a range of different shRNAs with a U6 small nuclear RNA promoter. The shRNAs used were targeted against different sites of the survivin gene, including exonic and intronic sequences, and were transfected into HeLa cells. The influence of survivin expression and cell apoptosis were both investigated. The present study may aid the development of a theoretical foundation for gene therapy in cancer.

## Materials and methods

**Cells line and culture.** The human cervical carcinoma HeLa cell line was provided by the Institute of Biochemistry and Molecular Biology of Guangdong Medical University (Zhanjiang, China). The HeLa cells were cultured in Dulbecco's Modified Eagles Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Zhejiang Tianhang Biotechnology Co., Ltd., Zhejiang, China), 100 U/ml penicillin, and 0.1 g/ml streptomycin (Beyotime Institute of Biotechnology, Shanghai, China) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Recombinant plasmid-based survivin-targeting shRNA design.** A total of 6 shRNAs targeting the survivin gene (and 1 negative control) were designed to be homologous to the survivin mRNA sequence (GeneBank no. NM001168). shRNA1 was targeted at intron 1; shRNA2 was targeted at intron 2; shRNA3 and shRNA4 were targeted at intron 3; shRNA5 was targeted at exon 1 and shRNA6 was targeted at exon 4 (Table I). According to BLAST analysis, the nucleotide sequences of survivin-targeted did not exhibit any non-specific interactions with other mRNA transcripts. All nucleotide sequences were synthesized and inserted into the recombinant plasmid vectors pGP-U6-GFP-Survivin by Shanghai GenePharma Co., Ltd. (Shanghai, China). The company presented a negative control shRNA (shNC), which did not possess any complementary region with survivin mRNA.

**Transfection of the shRNAs into HeLa cells.** Cells were seeded into 6-well plates in DMEM without antibiotics prior to transfection. When cells were at 60-70% confluence in monolayer, transfection of shRNA was performed using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol, with a non-transfected group acting as the blank control. The shRNA:lipid reagent was used at a ratio of 1:2; each shRNA was transfected into three marked wells, cultured in humidified incubator and the medium was changed with fresh medium each day.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** After a 48-h transfection, the total RNA of all groups was extracted using TRIzol reagent (Invitrogen;

Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. cDNA was synthesized from total RNA (1 µg) using the FastQuant RT kit (With gDNase) (Tiangen Biotech Co., Ltd., Beijing, China). qPCR was performed using the SYBR®Premix Ex TaqII kit (Takara Bio, Inc., Otsu, Japan) on LightCycler®480 (Roche, Switzerland). The human GAPDH gene served as an internal control. The primers used were: Survivin forward, 5'-TGACGACCCCATAGAGGAACA-3' and reverse, 5'-CGCACTTTCTCCGCAGTTTC-3'; and GAPDH forward, 5'-GGGTGTGAACCATGAGAAGT-3' and reverse, 5'-CAGTGATGGCATGGACTGTG-3'. The final qPCR volume was 20 µl, with 2 µl cDNA template and 0.4 µM of each primer. The qPCR parameters were as follows: Initial denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing and extension at 60°C for 20 sec. Results were analyzed in relation to GAPDH levels using the 2<sup>-ΔΔC<sub>q</sub></sup> method (25). Each experiment was performed in triplicate.

**Western blot analysis.** The total protein was obtained from each group using cold radio immune precipitation lysis buffer (cat. no. P0013B, Beyotime Institute of Biotechnology) containing phenylmethanesulfonyl fluoride (PMSF, catalog no. ST506-2, Beyotime Institute of Biotechnology) and protease inhibitors after a 72-h transfection. Protein concentration was quantified using the bicinchoninic acid (BCA) method using the Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. The proteins (60 µg/lane) were concentrated by 5% SDS-PAGE, separated by 12% SDS-PAGE and then transferred to a polyvinylidene fluoride membrane (cat. no. ISEQ00010, EMD Millipore, Billerica, MA, USA). The membrane was blocked using 5% skimmed milk in Tris-HCl Buffered Saline Tween-20 (TBST, 25 mM Tris-HCl, 125 mM NaCl, 0.1% Tween-20) at room temperature for 2 h with gentle agitation, and incubated overnight at 4°C with monoclonal anti-survivin (1:1,000; cat. no. 2808T; Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-GAPDH (1:1,000; cat. no. 2118S, Cell Signaling Technology, Inc.) antibodies. Following a wash with TBST, the membrane was incubated with mice anti-rabbit IgG-horseradish-peroxidase monoclonal antibodies (1:2,000; cat. no. 5127S, Cell Signaling Technology, Inc.) for 1 h at room temperature. The antibodies were diluted with 5% skimmed milk in TBST buffer. The protein bands were then visualized using ECL Chemiluminescence Detection kit (Beyotime Institute of Biotechnology), using GAPDH protein as reference. The density of the bands on the membrane was scanned with Canon Solution Menu EX (Canon, Zhanjiang, China) and analyzed with ImageJ 1.46 software (National Institutes of Health, Bethesda, MD, USA).

**FCM analysis.** A total of 4x10<sup>5</sup> HeLa cells were seeded in 6-well plants. All groups of cells were treated with EDTA-free trypsin (Beyotime Institute of Biotechnology) for 3 min after a 48-h transient transfection. Cells were then centrifuged at 425 x g at 4°C for 5 min and the supernatant was discarded. Cells were then washed with cold PBS and centrifuged at 425 x g at 4°C for 5 min, removed and supernatant discarded carefully, twice. Cells were re-suspended in 100 µl of 1X Binding Buffer, and 5 µl Annexin V-fluorescein isothiocyanate (FITC) and 5 µl

Table I. Sequences of survivin-shRNAs and negative control.

shRNA	Sequence length	Number of nucleotides
shRNA1	GGTGATGCTTACAACCTAA	19
shRNA2	GGGAGAGAGAAGGTGCTAA	19
shRNA3	GCTCATGCTTTCCTTGCTA	19
shRNA4	GCATTGGGCGCTGATTCTT	19
shRNA5	CCGCATCTCTACATTCAAGAA	21
shRNA6	GCACCACTTCCAGGGTTTATT	21
shNC	GTTCTCCGAACGTGTCACGT	20

shRNA, short hairpin RNA; shNC, negative control shRNA.

propidium iodide (PI) staining solution (cat. no. A221-01/02, all Vazyme Biotech Co., Ltd., Nanjing, China) were added, the solution was mixed gently and incubated at room temperature for 10 min in darkness. Next, 400  $\mu$ l of 1X Binding Buffer was added. The ratios of apoptotic cells were assessed using a Coulter EPICS XL Flow Cytometer using Expo32-ADC v. 1.2B software (Beckman Coulter, Inc., Brea, CA, USA).

**Hoechst 33258 stain analysis.** In order to access nuclear condensation by Hoechst 33258 staining,  $2 \times 10^5$  HeLa cells per well were washed twice with 2 ml PBS 48 h after transfection. A total of 1 ml Hoechst 33258 reagents (Beyotime Institute of Biotechnology) were added to each well and cells were incubated at 37°C for 30 min in the dark. Hoechst 33258 reagents were then removed and the cells were washed with PBS three times, 5 min each time. Morphological changes of apoptotic cells were observed under an inverted fluorescence microscope and images were captured.

**Statistical analysis.** SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) software was used for statistical analysis. The results were presented as the mean  $\pm$  standard deviation (SD) of three independent experiments. One-way analysis of variance was used to perform statistical comparisons of the data.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Expression of survivin mRNA following transfection of HeLa cells with shRNAs.** Survivin mRNA expression was evaluated in HeLa cells by RT-qPCR following transfection with recombinant plasmid vector-mediated survivin shRNAs (Fig. 1). For all groups treated with survivin-shRNA, survivin mRNA expression was significantly reduced compared with the blank control and shNC ( $P < 0.05$ ). No significant differences were observed in survivin mRNA expression between the shNC and the blank control groups ( $P = 0.09$ ). However, the effect of the suppression for the group transfected with shRNA1 was not well enough compared with other groups, and therefore it was not used in the following experiments.

**Expression of survivin protein following shRNAs transfection in HeLa cells.** Survivin protein expression was examined by western blot analysis (Fig. 2). Survivin protein levels in all

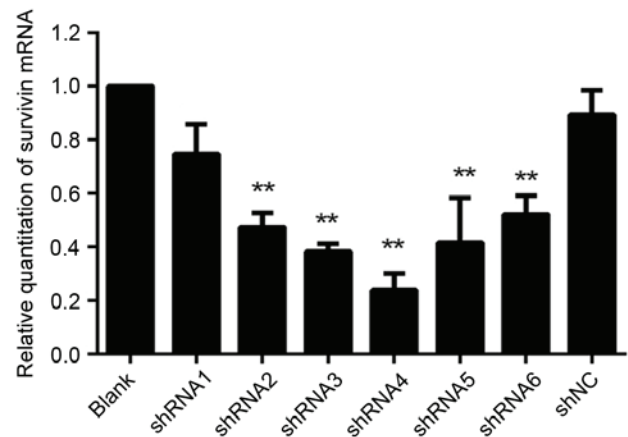


Figure 1. Quantitative representation of survivin mRNA levels. shRNAs were transfected into HeLa cells for 48 h and survivin mRNA levels were quantified by reverse transcription-quantitative polymerase chain reaction. Data shown are from three independent experiments. \*\* $P < 0.01$ , compared with the blank control. shRNA, short hairpin RNA; shNC, negative control shRNA.

shRNA-treated groups were significantly reduced ( $P < 0.05$ ; Fig. 2B); however, no significant differences were observed between the shNC-treated and blank-control groups ( $P = 0.40$ ). The expression of survivin protein can not only be reduced by RNA1 exon-specific shRNAs but also inhibited by reduced intron-specific shRNAs in HeLa cells.

**Effect of shRNA treatment on apoptotic rates, assessed by flow cytometry (FCM).** Cells transfected with shRNAs exhibited a significant increase in the level of apoptosis, as detected by FCM analysis via Annexin V-FITC/PI staining (Fig. 3; Table II). All data were presented as the mean  $\pm$  SD of three independent experiments. The apoptosis rates were significantly increased in the shRNA-treated groups compared with shNC-treated and blank-control groups ( $P < 0.05$ ). No significant differences were observed between the shNC-treated and blank-control groups.

**Effect of shRNA treatment on morphological changes of apoptotic cells by Hoechst 33258 stain analysis.** HeLa cells treated with shRNAs exhibited the following typical apoptotic changes following transfection (Fig. 4): Cell shrinkage, nuclear condensation and dense staining with some white color

Table II. Cell apoptosis rates after a 48-h transfection.

Groups	Apoptosis rate, %
Blank control	3.5±0.87
shNC	5.67±1.15
shRNA-2	27.63±1.59 <sup>a</sup>
shRNA-3	25.77±0.83 <sup>a</sup>
shRNA-4	37.87±2.25 <sup>a</sup>
shRNA-5	35.27±1.76 <sup>a</sup>
shRNA-6	28.5±2.91 <sup>a</sup>

Data are expressed as the mean ± standard deviation of three independent experiments, <sup>a</sup>P<0.01 vs. blank control. shRNA, short hairpin RNA; shNC, negative control shRNA.

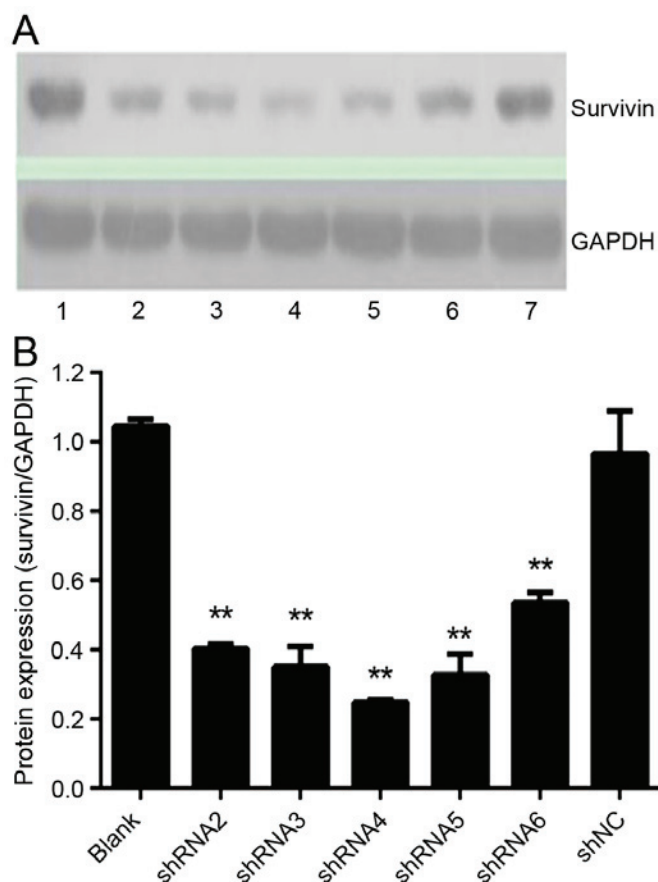


Figure 2. Effect of shRNAs on survivin protein expression. Protein levels were analyzed by western blot analysis after a 72-h transfection. (A) Bands of survivin and GAPDH were scanned. Lane 1, blank control; lane 2, shRNA2; lane 3, shRNA3; lane 4, shRNA4; lane 5, shRNA5; lane 6, shRNA6; lane 7, shNC. (B) The quantitative representation of survivin protein levels were determined by the density of the bands and one representation of three independent experiments was demonstrated, <sup>\*\*</sup>P<0.01 vs. blank control. shRNA, short hairpin RNA; shNC, negative control shRNA.

were observed under an inverted fluorescence microscope. Fluorescence staining in the group treated with shNC and the blank control group was light and uniform, and the number of apoptotic cells was markedly decreased.

## Discussion

The capability of cells to escape apoptosis is one of the defined hallmarks of cancer (26,27). Multiple previous reports indicated that survivin serves a critical function during tumorigenesis by inhibiting apoptosis, accelerating the progress of mitosis and promoting the growth of tumor cells (28,29). High survivin expression is observed in the majority malignant tissues yet is absent in mature, healthy tissues (3), indicating it may represent a promising therapeutic target. Various strategies have been taken to inhibit the expression of survivin in cancer cells, including the use of antisense oligonucleotides (30), dominant-negative mutants (31), ribozymes (32,33) and anti-cancer vaccines (34). RNAi may be a powerful tool for cancer therapy (35). RNAi is efficient at lower concentrations for anti-cancer treatment and results in fewer side effects compared with other techniques (35,36). RNAi-mediated inhibition of survivin expression has been used to delay mitosis by causing chromosome misalignment and prometaphase accumulation in HeLa cells (19,37), and has successfully reduced survivin expression (26,38-40).

The vast majority of human genes are made up of introns. They have been considered to be 'junk DNA' because they are degraded following splicing (41-43). However, a previous study demonstrated that introns serve a critical role in transcriptional regulation (44). Introns are not only involved in the formation of microRNAs and small nucleolar RNAs, but also regulate alternative splicing and affect the mRNA level (45). In the present study, the shRNAs targeting exons and introns effectively inhibited the expression of survivin in HeLa cells and increased the apoptosis rate, inducing marked apoptotic morphological changes. This indicates that introns were not 'junk DNA'.

The survivin gene contains four exons and three introns, and the survivin protein is the smallest member of the mammalian IAP family (2). To the best of our knowledge, few reports concerning the use of shRNA/siRNA targeted against introns to silence oncogenes exist, as the siRNA/shRNAs generated tend to target exons or promoters. As such, shRNAs in the present study were designed to block survivin expression by targeting exons and introns. A U6-promoter-mediated shRNA recombinant plasmid (pGP-U6-GFP-neo-Survivin) was utilized to inactivate survivin at multiple sites via multiple shRNA sequences. These plasmids were transfected into HeLa cells to evaluate the effectiveness of the survivin gene *in vitro*. All the shRNAs generated significantly downregulated expression of the target gene and protein, as assessed by RT-qPCR and western blot analysis. Notably, both FCM detection and Hoechst 33258 staining revealed an increased degree of apoptosis in HeLa cells induced by survivin inactivation.

RNAi is an evolutionary conserved regulatory mechanism by which siRNAs induce the cleavage and degradation of homologous mRNA molecules specifically by transcriptional gene silencing (TGS) and post-TGS and (15). The shRNAs that target exons can decrease expression of survivin mRNA and protein in the cytoplasm; however, the intron-specific shRNAs cannot find the homologous mRNAs in cytoplasm, so they cannot have mediated their inhibitory role in this way. Previous reports have demonstrated that RNAi can suppress gene expression through TGS in



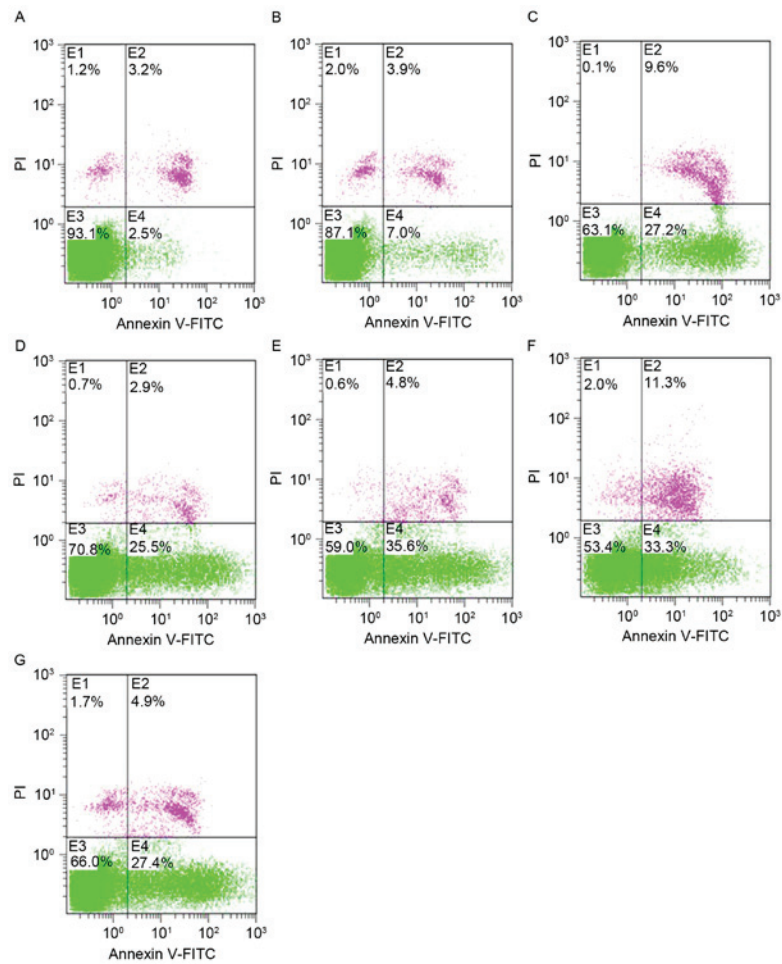


Figure 3. Effect of shRNAs on cell apoptosis in HeLa cells by flow cytometry analysis. The E1 quadrant was the cell debris, the E2 quadrant was the necrotic cell group, the E3 quadrant was the surviving cell group and the E4 quadrant was the apoptotic cell group. (A) Blank control; (B) shNC; (C) shRNA2; (D) shRNA3; (E) shRNA4; (F) shRNA5; and (G) shRNA6. The increase in the number of apoptotic cells was marked. shRNA, short hairpin RNA; shNC, negative control shRNA; FITC, fluorescein isothiocyanate.

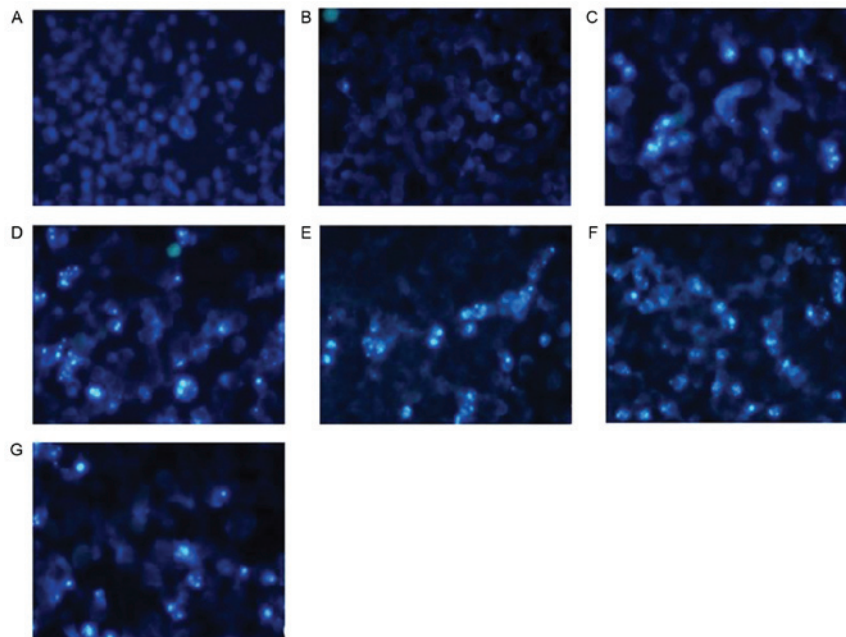


Figure 4. Effect of shRNA treatment on morphological changes of apoptotic cells by Hoechst 33258 stain analysis. (A) blank control; (B) shNC; (C) shRNA2; (D) shRNA3; (E) shRNA4; (F) shRNA5; and (G) shRNA6. The cells treated with shRNAs exhibited changes typical of apoptotic cells following transfection: Cell shrinkage, nuclear condensation and dense stain with some white color. The fluorescence staining of the shNC and blank control groups was light and uniform.

plants (46,47), *Drosophila melanogaster* (48), *Caenorhabditis elegans* (49,50), and *Schizosaccharomyces pombe* (51). Many studies have revealed that RNAi-mediated TGS in mammalian cells does occur at the nuclear level, via RNA-directed DNA methylation, RNAi-mediated heterochromatin formation and the modification of histones (52-54). In the present study, we hypothesize that the shRNAs targeting introns may have blocked survivin gene expression through TGS.

Upon entry into the nucleus, shRNAs are processed by the microprocessor complex containing the RNase-III enzyme Drosha and DiGeorge Syndrome Critical Region 8 (55,56) into a form that can be recognized by exportin-5, a Ran-GTP-dependent nucleocytoplasmic transporter (57,58). The modified shRNAs are then exported from the nucleus into the cytoplasm by exportin-5. In the cytoplasm, the functional siRNAs are cleaved by Dicer in a complex with protein kinase R activator of transcription and Tar-RNA-binding protein. The siRNAs are loaded into Argonaute proteins (59,60), forming the pre-RNA-induced initiator of transcriptional gene silencing (pre-RISC) complex. With the assistance of partners, such as Dicer and Argonaute proteins (49,61), the pre-RISC complex translocates into the nucleus, where the RISC complex matures. Single stranded siRNAs directly bind to complementary DNA sequences, leading to DNA methylation, heterochromatin formation and the post-translational modification of histones, such as the trimethylation of Lysine 27 of histone H3 (H3K27me3), histone deacetylation, dimethylation of lysine 9 of histone H3 Lys9 (H3K9me2) (62,63). Several proteins may be involved in this model, including histone-lysine-methyltransferases, Histone-deacetylases, DNA methyltransferases, and histone protein 1.

Another alternative explanation for this experimental phenomenon is that exogenous siRNAs that target intron sequences may control alternative splicing, in a process similar to that of TGS (64). In this silencing pathway, the mature siRNAs directly recognize nascent pre-mRNAs by targeting intronic sequences, leading to heterochromatin formation (H3K9 dimethylation and H3K27 trimethylation) and DNA methylation by recruiting associated enzymes, subsequently modulating alternative splicing. As in TGS, Argonaute proteins, particularly Argonaute 1 (AGO1) and Dicer, are also involved in this mechanism (63). It is possible that once the siRNAs bind to the nascent pre-mRNAs with AGO1 and Dicer, they may cleave them directly to trigger transcriptional change. However, the other possible mechanisms cannot be excluded as explanations of the results of the present study. Whether the shRNAs target intron sequences to suppress survivin expression *in vitro* through the aforementioned mechanism remains unclear and will be revealed by further experiments.

In summary, the results of the present study demonstrate that the inhibition of survivin expression by shRNA may be a potential tool for cancer therapy and may provide further options for the design of interference target sites.

### Acknowledgements

The authors would like to thank Professor Hou and Professor Huang for their guidance and Li Guodong for assistance with statistical analysis. The present study was supported by the Science and Technology Program of Higher Learning

Institutions (Dongguan, China; grant nos. 200910815264 and 2012108102016).

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