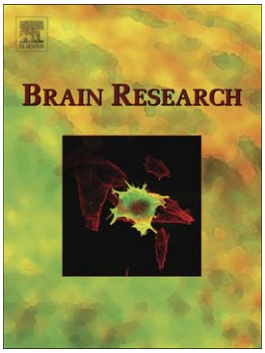

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Reduction of intersectin1-s induced apoptosis of human glioblastoma cells

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Abstract

Malignant gliomas have a high proliferation ability and high tendency to invade diffusely into surrounding healthy brain tissues, thereby precluding their successful surgical removal. Intersectin1 (also called ITSN1) as a molecular linker in the central nervous system is well known as an important regulator of endocytosis and exocytosis. ITSN1 has two isoforms: ITSN1-l and ITSN1-s. In this study, we show that siRNA-mediated down regulation of ITSN1-s induced glioma cells apoptosis. In addition, we demonstrate the possible mechanisms by which ITSN1-s functions in glioma cells apoptosis. Our data demonstrates that several key proteins, including FAK, Akt, Bcl-2, BAD which are critical for cells apoptosis were probably involved in ITSN1-s signaling pathways. Our results indicate that ITSN1-s is an effecter in regulation of gliomas cells apoptosis, and identify that ITSN1-s may be a new potentially anti-apoptosis target for therapeutic of gliomas.

Section:

1. Cellular and Molecular Biology of Nervous Systems

Key words: ITSN1-s; apoptosis; glioblastoma; Bcl-2; Akt

1. Introduction

ITSN1 is a highly conserved protein during evolution and possesses multiple domains. It is also called intersectin1, EHSH1, Dap160 or ESE-1 (Mafrica and Fodale, 2006). ITSN1 protein has two isoforms: the long isoform (ITSN1-l) and the short isoform (ITSN1-s). ITSN1-s isoform contains two EH domains, a coiled-coil KLERQ domain, and five SH3 domains. ITSN1-l contains DH domain, PH domain and C2 domain in addition to the domains shared with ITSN1-s (Gardiner et al., 2004). ITSN1 has the ability to bind protein-rich polypeptide and form complex with dynamin and synaptosomal-associated protein-23/25 (SNAP-23/25), which are important proteins for endocytosis and exocytosis (Keating et al., 2006).

Research has indicated that ITSN1 participates in both endocytosis and exocytosis, and regulates different intra-cellular signaling transduction pathways (Hussain et al., 2001; Scappini et al., 2007; Yamabhai et al., 1998). Our previous study reported that the cellular distribution of two isoforms ITSN1-l and ITSN1-s have their own specificity in the central nervous system. We found that ITSN1-l was highly enriched in neurons; while ITSN1-s was mainly detected in astrocytes and microglia (Ma et al., 2003). These results suggest that the expressions of ITSN1-l and ITSN1-s are strictly regulated in different cell types, and their unique cellular distribution should correspond to their functions.

In this study, we tested the hypothesis that ITSN1-s directly participates in glioma cells proliferation. LN-229 cells, in which ITSN1-s was down-regulated, exhibited significant induction of apoptosis. Furthermore, our data demonstrates that

several key proteins, including PAK1, FAK, Akt, Bcl-2, BAD which are critical for cells apoptosis were probably involved in ITSN1-s signaling pathways. Thus, our studies suggest that ITSN1-s is a regulator in glioma cells apoptosis, and may be a therapeutic target molecule for malignant glioma cells.

2. Results

First, plasmids expressing ITSN1-s siRNA sequences were transfected into LN-229 cells to obtain stable ITSN1-s down regulated LN-229 cells, which were designated as siITSN1/LN-229 cells. A siRNA vector containing a scrambled sequence was also transfected to the LN-229 cells to generate control cells, which were designated as scr/LN-229 cells. After the G418 selection, transfected cells were screened for ITSN1-s expression by Western Blotting. As ITSN1-l is specifically expressed in neurons, only ITSN1-s was detected in LN-229. The scr/LN-229 cells displayed similar ITSN1-s levels with the parental LN229 cells. ITSN1-s protein level was significantly reduced in siITSN1/LN-229 cells compared to control. As both ITSN1 and ITSN2 belong to ITSN family and the molecular weight of ITSN1-s is similar as ITSN2-s, we also detected ITSN2-s expression and found that siRNA interference of ITSN1-s did not alter ITSN2-s levels (Fig. 1A). Furthermore, we also detected the ITSN-1s expression in glioma tissue by immunohistochemistry. As shown in Figure1, ITSN-1s expression in glioma tissue was higher than the normal tissue.

In the following, we detected the rate of cell proliferation in both scr/LN-229 and siITSN1/LN-229 cells *in vitro*. The same number of scr/LN-229 and

siITSN1/LN-229 cells was seeded in culture plates at the same time, and the cells number were counted from the second day. The proliferation assay result suggested that the reduction of ITSN1-s led to decreased cell proliferation ($P< 0.05$) (Fig. 1B).

MTT assay was performed to detect the cells proliferation in scr/LN-229 and siITSN1/LN-229 cells which the result was shown in Fig. 2A. The data was measured at three time points 24 h, 48 h and 72h. The proliferation of siITSN1/LN-229 cells was greatly inhibited compared with the control. In the following, Annexin V-FITC apoptosis detection kit was applied to determine the cell apoptosis condition by flow cytometry. More apoptotic cells in siITSN1/LN-229 were observed shown in the Fig .2B, and the quantitive assay result showed a significant difference between the siITSN1/LN-229 and scr/LN-229 cells showing in Fig. 2C ($P< 0.05$).

TUNEL staining was performed to confirm the role of ITSN1-s in glioma cells apoptosis. LN-229 cells were transcient transfected with ITSN1-s siRNA, and ITSN1-s expression was greatly reduced 24 h after transfection compared with the control cells which was the same as in Figure 1A (data not shown). TUNEL assay was applied 24 h after transfection. More positive staining cells were observed in siITSN1/LN-229 cells group while less stained cells in scr/LN-229 cells group (Fig 3A). Such difference was significant which was analyzed in Fig 3B ($P< 0.05$).

As shown in above, we have found that reduction of ITSN1-s induced glioma cells apoptosis. Furthermore, how the ITSN1-s effects on glioma cells apotosis was determined in the following experiments. It was well known that the efflux of cytochrome C from mitochondria is the initial step leading to cell apoptosis (Piccotti

et al., 2002). Therefore, we detected the cytoplasmic cytochrome C level by flow cytometry. LN-229 cells were transcient transfected with ITSN1-s siRNA, flow cytometry was applied 24 h after transfection. siITSN1/LN-229 cells showed greatly higher level of cytochrome C compared with the scr/LN-229 cells (Fig. 4A) and the quantitive assay result showed a significant difference between the siITSN1/LN-229 and scr/LN-229 cells showing in Fig. 4B ($P< 0.05$). The level of cytoplasma cytochrome C was also examined by Western Blotting. siITSN1/LN-229 cells had higher level of cytochrome C than scr/LN-229cells, consistent with the flow cytometry result (Fig. 4C).

BAD, a pro-apoptotic protein of the Bcl-2 family, has been identified as a crucial factor of apoptotic signaling pathways in cancer cells (Piccotti et al., 2002). In this study, Bcl-2 and BAD protein expression was studied upon ITSN1-s reduction. siITSN1/LN-229 cells showed increased expression of BAD and decreased Bcl-2 expression compared with the scr/LN-229cells.

Focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase located in the focal adhesion complex, has been implicated to play anti-apoptotic role in cancer cells (Oliveira-Ferrer et al., 2008). Reduction of FAK phosphorylation induces apoptosis through down regulation of Akt survival signaling (Liao et al., 2005). In order to detect whether ITSN1-s participates in this apoptotic signaling pathway, phosphorylation of FAK and Akt combined with the down stream factor PAK1 were all examined. Fig 5 showed that reduction of ITSN1-s exerted a significant inhibition effect on activation of FAK, Akt and PAK1.

To determine if the vitro assays described above have any bearing on tumorigenicity *in vivo*, we applied the subcutaneous mouse xenograft model to validate the role of ITSN1-s in glioblastoma apoptosis *in vivo*. As the scr/LN-229 and LN-229 cells showed the similar function characteristic in the above cellular experiments (data not shown); scr/LN-229, instead of LN-229 cells were used in the *in vivo* assay. Stable clones of siITSN1/LN-229 and scr/LN-229 were subcutaneously injected in 10 Nu/Nu mice respectively. The tumor volume of siITSN1/LN-229 group showed great reduction compared with the control mice (Fig 6A, B).

3. Discussion

ITSN1 is regarded as an adaptor protein according that it has the ability to bind different kinds of proteins and can interact to each other by its various domains (O'Bryan et al., 2001). The EH domain of ITSN1 has been reported to interact with endocytic proteins, including secretory carrier membrane protein 1 (SCAMP1) and epsins/Ibps (Fernandez-Chacon et al., 2000); the coiled-coil KLERQ domain has been shown to bind EPs15 and SNAP25 (Gardiner et al., 2004); and the SH3 domain can interact with several proteins essential for endocytosis including dynamin, neural Wiskott-Aldrich syndrome protein (N-WASP), synaptojanin, and so on (Ma et al., 2003; Roos and Kelly, 1998; Sengar et al., 1999; Yamabhai et al., 1998).

ITSN1 has been widely studied in the central nervous system for its important function in endocytosis and exocytosis. Recent reports suggest that it also plays a critical role in signaling transduction pathways. Our previous study reported that the cellular and tissue distribution of two isoforms ITSN1-l and ITSN1-s have their own

specificity in the central nervous system (Ma et al., 2003). Northern blotting analysis reported that the ratio of mRNA of ITSN1-l to ITSN1-s expression in brain increased after birth (Pucharcos et al., 2001). Therefore, two isoforms of ITSN1 probably are strictly regulated in each cell or tissue type during development and play isoform-specific roles according to their different distribution.

In our study, we demonstrated that ITSN1-s directly participated in glioma cells apoptosis and the possible mechanisms. Our data demonstrated that several key proteins, including FAK, Akt, PAK, Bcl-2, BAD which are critical for cells apoptosis were probably involved in ITSN1-s signaling pathways. Our results indicated that ITSN1-s acts an effecter in regulation of gliomas cells apoptosis possibly through mitochondria and Akt survival signaling.

It has been reported that ITSN1-s regulates the mitochondrial apoptosis pathway in endothelial cell (Predescu et al., 2007). In the present study, we showed that reduction of ITSN1-s induced glioma cells apoptosis through enhancing cytochrome C efflux from mitochondria and inhibiting the expression of anti-apoptotic proteins, which was well consistent with the previous report. Margaret et al demonstrated that ITSN has the ability of binding to the subunit of PI3K and regulating neuron survival by PI3K/Akt pathway (Das et al., 2007). In this study, we found that the Akt signaling was also impaired with reduction of ITSN-1s. Thus, we speculate that ITSN1-s, as a ubiquitous isoform of ITSN1, functions on mitochondria and Akt survival pathways to regulate glioma cells apoptosis.

4. Experimental Procedures

Cell culture and reagents

Human glioblastoma LN229 cell line were obtained from American Type Culture Collection (Manassas, VA, USA) and were cultured in DMEM with 10% FBS (complete medium). The recombinant human epithelial growth factor (EGF) was obtained from R&D systems (Minneapolis, MN, USA). The antibodies toward rabbit anti-Akt, rabbit anti-phosphorylated Akt, β-actin, mouse anti-FAK, rabbit anti-phosphorylated FAK, mouse anti-Bad, rabbit anti-Bcl-2, goat anti-Intersectin2 were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies about rabbit anti-PAK1 were bought from Invitrogen Corporation and rabbit anti-phosphorylated PAK1 from Cell signaling Technology (Beverly, MA, USA), chicken anti-intersectin1 from Abcam (Cambridge, UK). Annexin V-FITC Apoptosis Detection Kit Plus was purchased from Biovision (Palo Alto, CA), FITC anti-Cytochrome C antibody was bought from Biolegend (San Diego, CA). One Step TUNEL Apoptosis Assay Kit was acquired from Beyotime (Beijing, China) and MTT agent was from Sigma (St. Louis, MO, USA).

Immunohistochemistry

Immunohistochemisty was performed using standard techniques. Human glioma tissues were obtained from Tianjin Medical University Cancer Institute & Hospital. The pathological diagnosis is Glioblastoma. Antigen retrieval was performed by autoclaving. Incubation with 10% serum in phosphate-buffered saline was performed for 15 minutes to eliminate nonspecific staining. Incubation with antibody was carried out. After washing unbound antibody, sections were treated with the horseradish

peroxidase labeled polymer (DAKO Envision System, DAKO Corporation). Immunohistochemical reactions were developed with diaminobenzidine as chromogen. Finally, sections were lightly counterstained with 10% Mayer hematoxylin, dehydrated, mounted, and observed.

Plasmid transfection

Transfection was performed as described as previously (Zhang et al., 2009). Cells were seeded in a 35-mm dish and cultuted for 24 hr before transfection in the complete medium. The transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. ITSN1-s specific siRNA plasmid for LN-229 cells (insert: GGCTGGCTTGGAGGAGAATTA inserted into pGPU6/GFP/Neo) and scrambled sequence scr/siRNA were from Genespharmer (Shanghai, China). In order to establish stable si/Intersectin1 transfected cell lines, the G418-resistant cells were screened, and their expression level of intersectin1 was monitored by Western blotting.

Transcient transfection

ITSN1-s siRNA (5'-UUUCAGUACAACUAUGUCCUU -3') were transfected into LN-229 cells by using GenePorter transfection reagents (Gene Therapy Systems, San Diego, CA, USA) according to the manufacturer's instructions and processed for immunofluorescence 24 hr after transfection.

Proliferation assay

Proliferation assay was performed as described as previously (Zhang et al., 2009). In brief, ITSN1-s silence cells and the control cells were plated in 6-well plates at

5×10^4 cells per well and cultured in a complete medium at 37℃ incubator complement with 5% CO_2. From the second day, ITSN1-s siRNA cells and control cells were trypsinized，the total cell number was counted every day and growth curves were constructed.

MTT assay

This assay detected the ability of viable cells to convert a soluble tetrazolium salt, MTT [3-(4.5- dimethylthiazol -2-yl)-2-5-diphenylterazolum bromide], into a blue formazan end product by mitochondrial dehydrogenase enzymes. Briefly, 10 μl of MTT stock (5 mg/ml of MTT in PBS) was added to each culture. After incubation for 4 h at 37ºC in an atmosphere of 5% CO2 and 95% humidity, 100 μl of 10% SDS in 0.01M HCl was added to each well. The solution was allowed to solubilize overnight in 100% humidity. The absorbance of each well was determined by using a scanning multiwell spectrophotometer ELISA (Bio-Tek Instruments Inc., VT) at 490nm.

TUNEL Assay

The TUNEL assay was carried out by One Step TUNEL Apoptosis Assay Kit. Cells were treated with or wothout transcient transfection of ITSN1-s siRNA, and 24 hr later cells were washed with PBS and fixed with 4% paraformaldehyde for 45 min at room temperature, then cells were treated with 0.1% TritonX-100 for 2 min on ice. The TUNEL reaction mixture (label and enzyme solutions), prepared as indicated by the instructions, was applied on cell monolayers for 60 min at 37℃ in a humidified atmosphere in the dark. TUNEL-positive cells were identified and counted under a fluoresence microscope at 200 ×.

Apoptosis Detection

Annexin V-FITC apoptosis detection kit was applied to determine the cell apoptosis condition by flow cytometry. Cells were treated with or without transcient transfection of ITSN1-s siRNA, 24 hr later cells were digested with trypsogen. After that 5×10^5 cells were collected by centrifugation, and then were resuspended in 500μl of Binding Buffer, 5μl of Annexin V-FITC and 1μl of SYTOX Green dye were added in the Binding Buffer and cells were incubated at room temperature for 10 min in the dark. The apoptotic cells were quantitatively detected by flow cytometry.

Cytochrome C detetction by flow cytometry

Cells were treated with or without transcient transfection of ITSN1-s siRNA, and 24 hr later cells were washed 3 times with cold PBS, and then were digested with trypsogen and centrifuged at 1000 rpm for 5min, cell were then suspended in 500μl of PBS. After that, the cells were incubated with FITC anti-Cytochrome C antibody at room temperature for 60 min in the dark and Cytochrome C efflux from mitochondria was detected by flow cytometry.

Western blotting

Western blotting was performed as described as previously (Zhang et al., 2009). The cells were lysed by 1×SDS lysis buffer (Tris-HCl, pH 6.8, 62.5 mM, 2% SDS, 10% glycerol) followed by centrifugation at 10,000 rpm for 10 min at 4℃. Equal amounts of cell lysates (20 -40μg total protein/lane) were loaded and separated by SDS-PAGE, and proteins were transferred onto nitrocellulose membranes (Immobilon-P, Millipore, Billerica, MA, USA), probed with anti-Akt (1:1000), P-Akt

(1:1000), PAK1 (1:1000), P-PAK (1:0000), FAK (1:500), P-FAK (1:1000), Bad (1:1000), Bcl-2 (1:500), Cytochrome C (1:1000), and β-actin (1:5000) followed by HRP-conjugated or AP-conjugated secondary antibodies.

Tumorigenicity Assay

Male athymic Nu/Nu mice, 4 to 5 weeks old, were purchased from Wei Tong Li Hua Experimental Animal Co. Ltd. (Beijing, China). Scr/LN-229 or siITSN1/LN-229 cells were injected into mice respectively. In detail, a total of 3×10^6 cells were subcutaneously inoculated into one flank of each mouse. The sizes of tumors were measured each week. All animals received human care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals, prepared by the National Academy of Sciences and published by the National Institutes of Health. Experimental group (n=10) and control group (n=10) of mice were used.

Statistic analysis

Statistical analysis was carried out using Prizm 3 from GraphPad Software (San Diego, CA). Data are presented as mean ± SD. Statistical significance for comparisons between groups was determined using Student's paired two-tailed t-test. All the results were generated from three independent experiments.

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Figure Legends

Figure 1: The establishment of stable LN-229 cells expressing disrupted ITSN1-s. (A) LN-229 cells were transfected by siRNA ITSN1-s plamid or control plasmid, stable clone was screened by G418 selection. The expression of ITSN1-s and ITSN2-s in LN-229 cells was examined by Western Blotting. β-actin was used as a loading control. Immunohistochemisrty results of ITSN-1s expression in glioma tissue (b) and normal tissue (a) (100×). (B) Comparison of cell proliferation in scr/LN-229 and siITSN1/LN-229 cells. Each data point was an average of triplicate assays. (Bars, standard deviation; Two-way ANOVA analysis, $P < 0.05$).

Figure 2: Reduction of ITSN1-s induced apoptosis of glioma cells. (A) MTT assay was performed as described in "Materials and Methods". Stable cell clones same as in Figure 1 were used. OD was measured at various time points: 24 h, 48 h, 72 h. (B) Annexin V-FITC apoptosis detection kit was used for apoptosis assay by flow cytometry. Apoptotic condition of cells was showed in the images. (C) Quantitative result of apoptosis assay by flow cytometry was analyzed. (Two-way ANOVA analysis, $P < 0.05$)

Figure 3: The role of ITSN1-s in regulating glioma cells apoptosis was confirmed by TUNEL assay. (A) The TUNEL assay was carried out by One Step TUNEL Apoptosis Assay Kit. LN-229 cells were treated with or without transcient transfection of ITSN1-s siRNA. The images of TUNEL positive cells were captured by a fluorescence microscope 24 h after transfection (100×). (B) Quantitative result of TUNEL assay was analyzed. (Two-way ANOVA analysis, $P < 0.05$)

Figure 4: The role of ITSN1-s in regulating glioma cells apoptosis was further

confirmed by measuring cytochrome C efflux from mitochondria. (A) LN-229 cells were treated with or without transcient transfection of ITSN1-s siRNA. Cells were incubated with FITC anti-Cytochrome C antibody at room temperature in the dark and cytochrome C efflux from mitochondria was detected by flow cytometry 24 h after transfection. (B) Quantitative result of assay was analyzed. (Two-way ANOVA analysis, $P < 0.05$) (C) Protein level of cytochrome C, BAD and Bcl-2 were determined by Western Blotting. Result is the representative image from three independent experiments.

Figure 5: Activation of FAK, AKT and PAK1 were detected with the reduction of ITSN1-s. Stable cell clones same as in Figure 1 were used. EGF was used to stimulate the cells for various time points: 0 min, 1 min, 5 min, and 15 min. Then cells was lysed and prepared for Western Blotting. Result is the representative image from three independent experiments.

Figiure 6: Reduction of ITSN1-s induced glioma cells apoptosis *in vivo* assay. (A) Stable clones of scr/LN-229 and siITSN1/LN-229 were subcutaneous injected into Nu/Nu mice respectively. The sizes of tumors were measured each week. 6 weeks later, the representative images of tumor size in each group were captured. (B) Quantitative result of *in vivo* assay was analyzed. (Two-way ANOVA analysis, $P < 0.05$)

Fig1

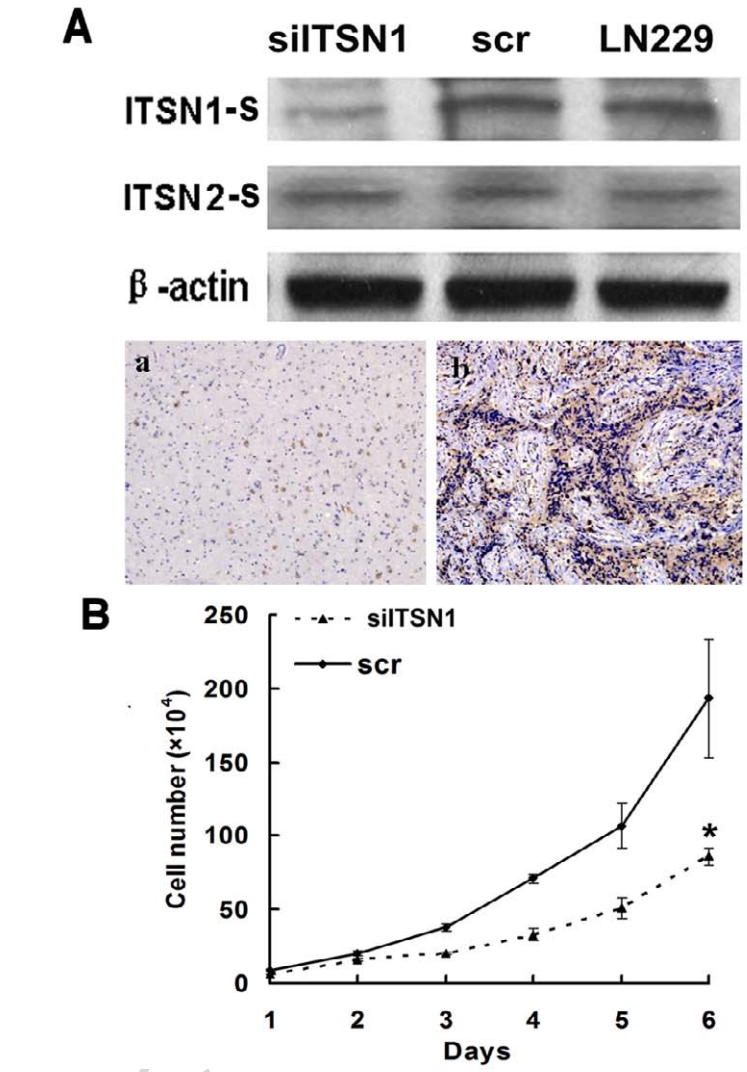
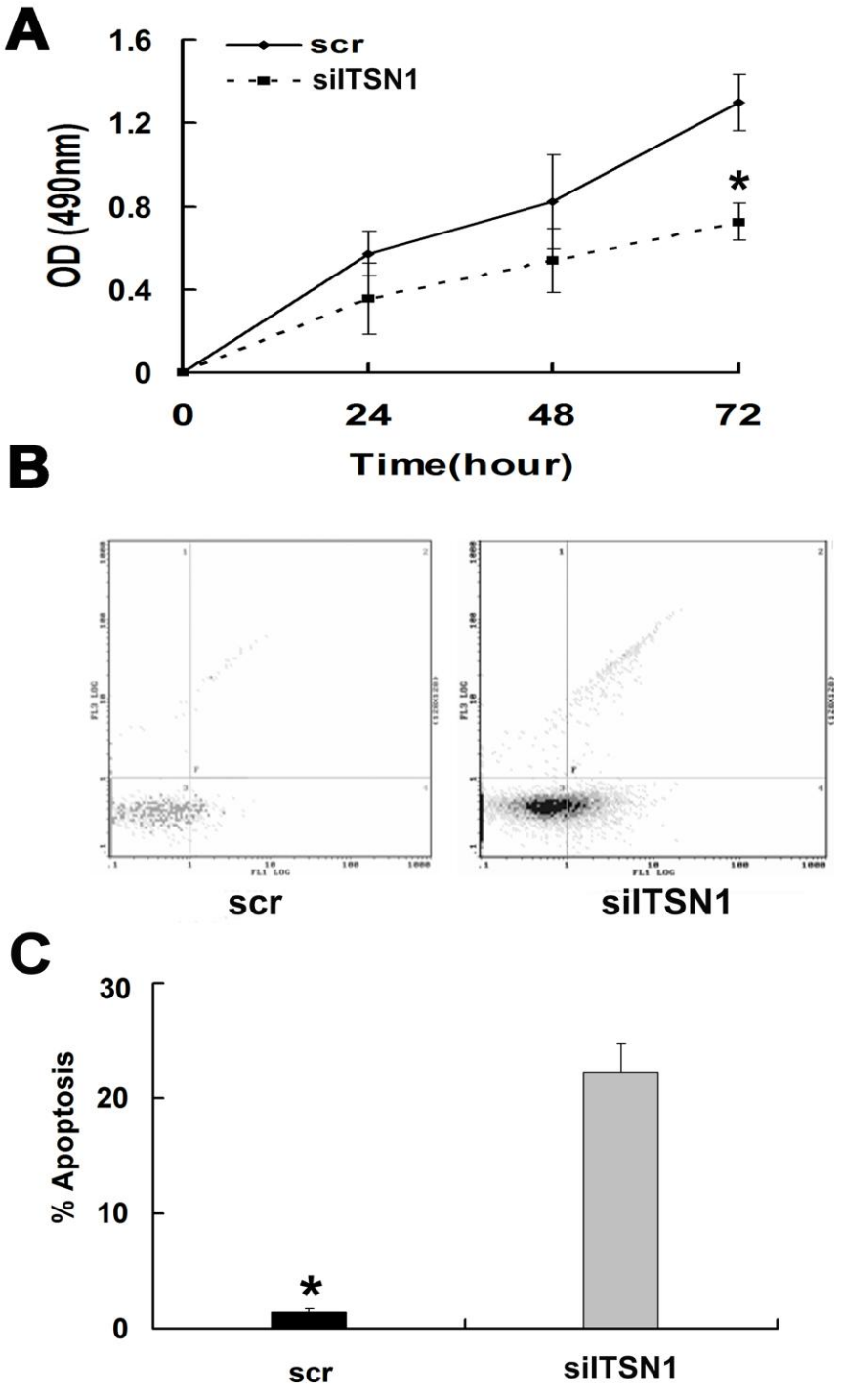


Fig2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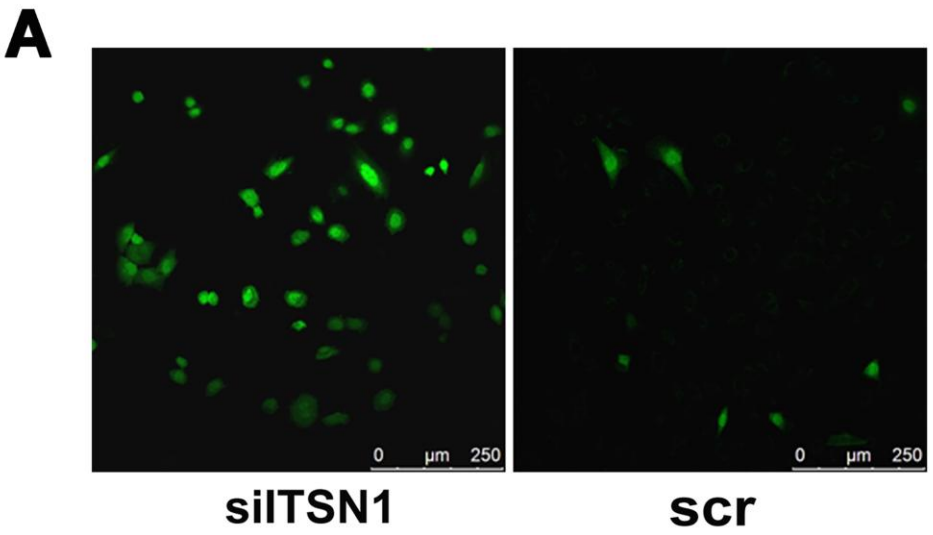


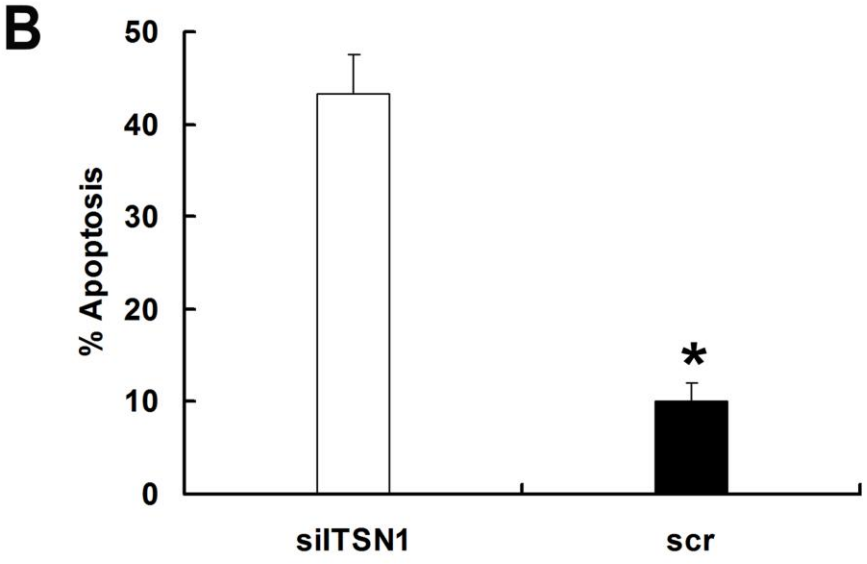


Fig4

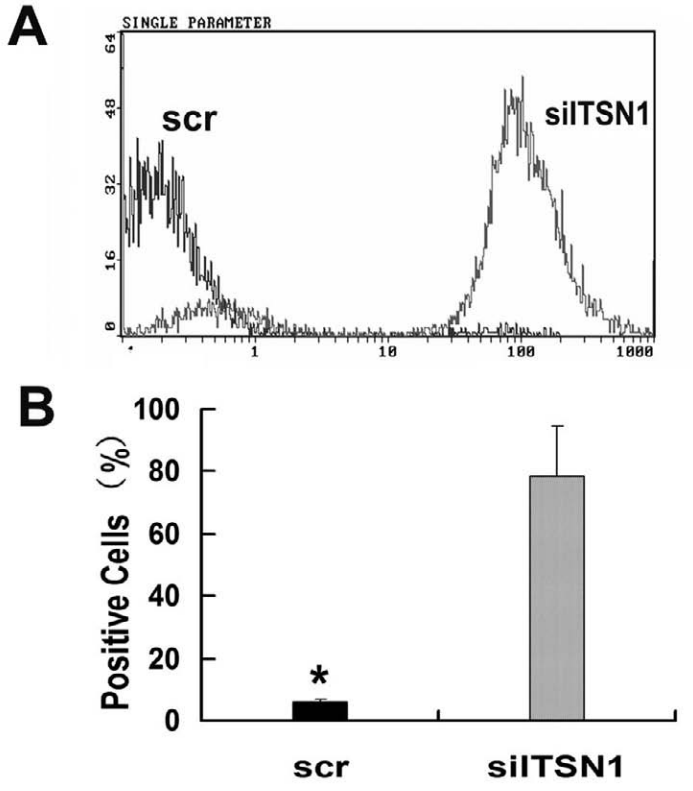


Fig5

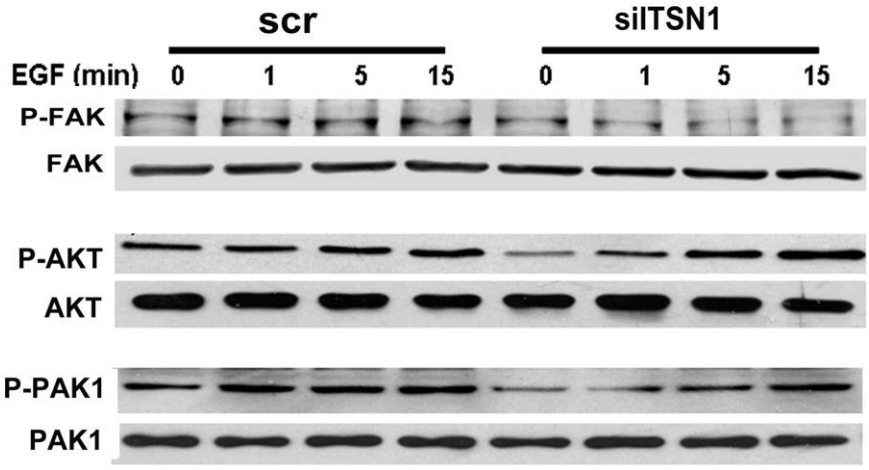
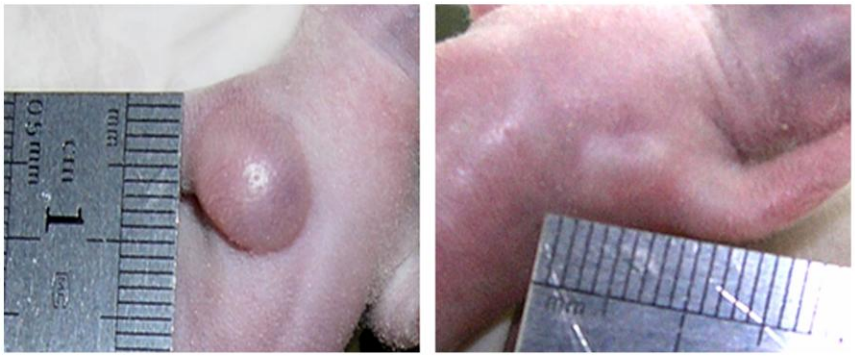


Fig6

A



B