RESEARCH ARTICLE

Apoptotic Effects of psiRNA-STAT3 on 4T1 Breast Cancer Cells *in Vitro*

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Abstract

Background: The aim of this study was to investigate the effect of a Lipofectamine2000 (Life2000) Transfection Reagent transfected psiRNA-STAT3 plasmid on 4T1 breast cancer cells. Materials and Methods: MTT was used to detect the cell proliferation of breast cancer 4T1 cells at different periods (0h, 6h, 8h, 10h); the cell cycle was assessed by flow cytometry; variation of apoptosis and mitochondrial membrane potential was observed under a fluorescence microscope; immunohistochemical staining was used to determine the expression of caspase-3 and cyclin-D1 protein. Results: An obvious effect of inhibition to 4T1 cancer cells could be observed at 8h after the psiRNA-STAT3 was transfected. Typical alterations of apoptotic morphological features were visible in the psiRNA-STAT3 treatment group. Mitochondrial membrane potential decreased significantly, the number of cells was increased in G0/G1 phase, and the number of cells was decreased in S phase, and the data were statistically significant ($p<0.05$), compared with the Scramble and Mock groups. Expression of caspase-3 protein was increased significantly, while that of cyclin D1 was significantly decreased. Conclusions: Life2000 transfected psiRNA-STAT3 plasmid can inhibit 4T1 tumor cell proliferation and promote apoptosis of 4T1 tumor cells, which process depends on the regulation of expression of cyclin D1 and caspase-3 protein.

Keywords: psiRNA-STAT3 plasmid - breast cancer 4T1 cells - apoptosis - protein expression

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Introduction

Breast cancer is a malignant tumor which causes serious damage to the women's health (Wang et al., 2012; Reynoso-Noveron et al., 2013). Its incidence has become the first (Canzian F et al., 2010) malignant tumor in the women. The etiology of breast cancer includes genetic disease, lifestyle, and dietary factors. Gene therapy is a new therapy after operation, chemotherapy and endocrine therapy, which has better targeted, more suitable for the implementation of individualized treatment of breast cancer patients, compared with the traditional treatment model. In recent years, with deeper understanding of tumor molecular pathology and the rapid development of the gene therapy of breast cancer, some targeted drugs have been used successfully in clinic and achieved good effect (Dao et al., 2014). Studies have shown that signal transduction and activation of transcription factor STAT3 is the focus of a plurality of carcinogenicity channel aggregation, with persistent and excessive activation in breast cancer (Tkach et al., 2013), The continuous activation of STAT3 is characteristic of breast cancer. STATS signaling pathway disorder on the formation of breast tumors is very important (Hsieh et al., 2005; Aggarwal et al., 2006; Zhang et al., 2007). Therefore, STAT3 is a hotspot in gene therapy of tumor. Our previous results confirmed that attenuated Salmonella plasmid carrying psiRNA-STAT3 has obvious inhibiting effect on mouse breast cancer, and explored the mechanism of preliminary study (Zhou et al., 2012). This study was designed to investigate the effects of Lipofectamin2000 transfected psiRNA-STAT3 plasmid on the proliferation, apoptosis, cell cycle and related Cyclin-D1 and Caspase-3 protein expression, for its clinical application to the breast cancer treatment.

Materials and Methods

Materials

Breast cancer cell lines 4T1 cell lines were purchased from Shanghai Institute of Cellular Biology of Chinese Academy of Sciences.

Plasmids psiRNA-STAT3 (human mouse homologue) and psiRNA-Scramble which were provided by the Department of pathophysiology, Basic Medical College of Jilin University

Reagents (1). Opti-MEM culture medium (GIBCO, USA, product number: 1267487); (2). Lipofectamine2000 Ttansfection Reagent (Invitrogen by life technologies, USA, product number: 1345247); (3). Detection of

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mitochondrial membrane potential test kit (JC-1, serial number: C2006), cell cycle and apoptosis detection kit (serial number: C1052), DAB horseradish peroxidase color development kit (serial number: C0202) by Beyotime Institute of biotechnology, Jiangsu, China (4). Rabbit Anti-Cyclin D1 antibody and Rabbit Anti-Caspase-3 antibody (Boster, wuhan, China). (5). The ethidium bromide (Sigma, USA Lot : E8751:); (6). Acridine orange (Ding Guo Changsheng Biotechnology Co., Ltd.Beijing, China Lot:1AB10220); (7). Dimethyl sulfoxide, methyl thiazolyl tetrazolium (MTT) (Sigma, USA)

The main instruments

(1). The fluorescence microscope NiKon ECLIPSE 80i (Nikon, Japan); (2). CO_2 incubator (Sanyo manufacturing Co., Japan, model: NCO-15AC); (3). AN YANG super clean bench (AnyangTechnology Development Co., Ltd.Suzhou, China, model: BSC-BOO II B2); 4). flow cytometry (Becton-Dickson, USA).

Methods

Different concentrations of Lipofectamin2000 Ttansfection Reagent transfected psiRNA-STAT3 plasmid

Set 4 EP tubes, labeled A, B, C, D respectively, each tube was added with 550μL Opti medium. A and B were added with 8.8μL psiRNA-STAT3 and psiRNA-Scramle. C and D were respectively added with 22μl Life2000 transfection reagent. Mix the C tube with the A tube, and mix D tube with B tube at room temperature for 20min., the mixture prepared above were added to psiRNA-STAT3 group and psiRNA-Scramble group, gently jiggled.

The cell viability was detected by MTT

(1). The logarithmic growth phase of breast cancer 4T1 cells, 0.25% trypsin, counting cells, cell suspension of 1×10^4 cells/mL, each hole was added with 100μL cell suspension, which was seeded into 96 well plate, at 37°C, 5% CO_2 incubation (2). randomly divided for Mock group, psiRNA-Scramble group and psiRNA-STAT3 group.Each group had 6 holes., according to the grouping administration (reference 2.1) (3). The cells growth were detected at 6, 8 and 10 hours respectively (4). At each time point, added with 20μL MTT solution of 5mg/mL, incubated for 4h, the supernatant was discarded, then added with DMSO 150 μ L. Cells were shocked for 10min, aiming to fully dissolve the crystals (5). Enzyme immunoassay instrument to detect the absorbance (OD), set absorption wavelength at 490nm.

Acridine Orange / Ethidium Bromide (AO/EB) double staining

(1). The cell climbing piece breast cancer 4T1 cells in logarithmic growth phase, 0.25% trypsin. Adjusted the cell suspension to 3×10^5cells/mL, and inoculated in six-well plate, 37°C, 5%CO_2 for 4 hours. (2). Cells were randomly divided into Mock group, psiRNA-Scramble group and psiRNA-STAT3 group, each group had 2 holes, grouped by the administration (reference 2.1). The final volume of each hole is 2mL, 37°C, 5%CO_2 for 8 hours. (3). Removed coverslips covered with cells, then placed on a slide, dropwise addition of 5μl dye AO and EB on coverslips. Observed immediately under the fluorescence microscope, photograghed. Counted the number of programmed cell death of 100 cells per field, the number of programmed cell death were calculated.

Detection of mitochondrial membrane potential (JC-1)

(1). breast cancer 4T1 cells of the logarithmic growth phase, 0.25% trypsin digestion, inoculated in 6 well plate with 3.0×105cells/mL, the experiments were divided into Mock group, positive control group, psiRNA-Scramble group, psiRNA-STAT3 group. Each group had 2 holes, discarded broth until cells were adherent. (2) Added 10% medium without double-resistance, grouped by the administration (reference 2.1), the final volume was 2mL, incubated for 8 hours;37°C, 5%CO_2 (3). when transfected for eight hours, positive control group of each hole added CCCP (CCCP according to the ratio of 1:1000 was added to the cell culture medium). The psiRNA-Scramble and psiRNA-STAT3 group each hole was added with 0.5mL JC-1 staining liquid. After incubation for 20 min at 37°C, discarded supernatant, and cells were rinsed 2 times with JC-1 (1X) buffer of ice bath, each well was added 1mL culture medium (4). Observed under the fluorescence microscope, the excitation wavelength of 490nm, emission wavelength of 530nm, photographed. Counted the number of programmed cell death of 100 cells per field, the number of programmed cell death was calculated.

The cell cycle was detected by flow cytometry

(1). Breast cancer 4T1 cells in logarithmic growth phase, 0.25% trypsin digestion. Adjusted the cell suspension to 1×10^6cells/mL, then inoculated in six-well plates. Experiments were randomly divided into three groups, Mock group, psiRNA-Scramble group, psiRNA-STAT3 group, each group with two wells (2). Arranged in 37°C, 5%CO_2 for 8 hours to culture, with 0.25% trypsin digestion, cells were collected, then centrifuged 1000rpm/min for 5 minutes, discarded the supernate, resuspended cells gently with 200μL PBS; (3). Then, added the following components: (Rnasa, Pyridine iodide (PI), Triton-100 was added with 80μL respectivly). protected from light for 15min. (4). Detected on the machine .The excitation wavelength is 488nm, calculated the apoptosis rate and analyzed cell cycle by software.

SABC immunohistochemical assay

(1) Dewaxed with xylene and hydrated with alcohol of gradient (2). Antigen repaired, the slices were immersed in 0.01M citric acid buffer (pH6.0) (3). Inactivation of endogenous peroxidase, at room temperature for 5-8 minutes (4). Normal serum blocking solution (5%) was placed at room temperature for 20 minutes. (5). Added the detection antibody to the specimen (according to Cyclin D1, 1:300, Caspase-3, 1:100 concentration dilution), then placed them into 4°C refrigerator overnight and washed with PBS. (6). Added biotin-labled conjugated antibody of mouse to the specimen, and incubated for 20 minutes, 37°C.Then, HRP- labeled streptavidin was dropped into the specimen, which were incubated for 20 minutes, 37°Cbefore washed with PBS. (7). DAB developer was dropped into the specimen, developping at room temperature. (8). Counterstained with hematoxylin, until

water was from back to blue. Dehydrated with gradient alcohol, and xylene was transparent. Mounted the slices with neutral resin, then observed under the microscope, photographed.

Results

The effect of Life2000 transfected psiRNA-STAT3 plasmid on proliferation of breast cancer 4T1 cells

The breast cancer 4T1 cells were treated for 0, 6, 8, 10h, and then observed under the microscope.Cells grow well and distributed symmetrically at 0h.6 hours after transfection, cells in MOCK group continue to grow, the cell number increased; psiRNA-Scramable group grow slow, and the number of cells reduced; psiRNA-STAT3 group cell number decreased more obviously. 8 hours after transfection, cells in MOCK group continued to increase while cells number decreased in psiRNA-Scramble group; the number of cells continued to reduce in psiRNA-STAT3 group, and a large number of cells floated in the culture medium.10 hours after transfection, cells in MOCK group continued increasing; the number of cells in psiRNA-Scramable group and psiRNA-STAT3 group were higher than 8h. (Figure 1)

The alterations of mitochondrial membrane potential and apoptosis by AO/EB double staining of 4T1cells treated with life2000 which were transfected into psiRNA-STAT3 plasmid for 8h

AO/EB double staining. cells in Mock group were polygonal, with projections, and the nuclear chromatin was bright green, a few early apoptosis cells can be detected occasionally (Figure 2, A-1), 8h after transfection, the early apoptosis and late apoptosis cells increased obviously in psiRNA-Scramble (Figure 2, A-2); The psiRNA-STAT3 group cells changed from polygonal to rounded, and there are many green and circular nuclear chromatin or orange and pyknotic nuclear chromatin (Figure 2, A-3). Positive control group treated with CCCP for 20min showed green fluorescence (Figure 2, B-1); In psiRNA-Scramble group, cells were in good condition, showed red fluorescence a lot, a little green fluorescence (Figure 2, B-2); Cells in Mock group were in good state, showed red fluorescence, cytoplasm was stained deeply, and a few apoptotic cells were detected (Figure 2, B-3); psiRNA-STAT3 group showed many green fluorescence, a little red fluorescence (Figure 2, B-4).

The results showed that: alteration from red fluorescence to green fluorescence is a marker of early apoptosis .Transfection for 8h, psiRNA-STAT3 plasmid had significant influence of apoptosis induction on 4T1 cells .psiRNA-STAT3 group has significant difference, compared with Mock group, psiRNA-Scramble group ($p<0.05$). psiRNA-Scrambl group showed no significant difference, compared with Mock group ($p>0.05$). (Table 2)

Effect of psiRNA-STAT3 transfeted with Life2000 for 8h on the cell cycle of 4T1 cells

The changes of cell cycle were detected by FCM marked with PI.Results showed that, the number of

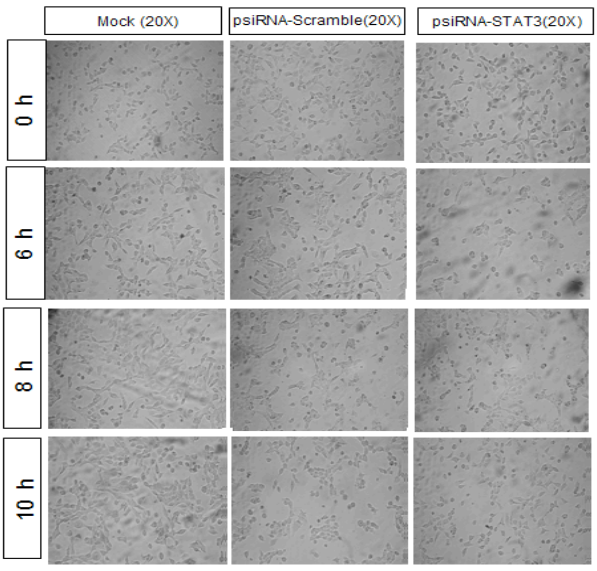

Figure 1. The Effect of Life2000 Transfected psiRNA-STAT3 Plasmid on Proliferation of Breast Cancer 4T1 Cells for 0, 6, 8, 10h

Table 1. Effects of siRNA on Cell Growth (n=6, $\bar{X}$±S)

Group	OD		
	6h	8h	10h
Mock	0.20217±0.041	0.20500±0.025	0.20017±0.033
psiRNA -Scramble	0.19250±0.022	0.19767±0.017#	0.19083±0.037
psiRNA -STAT3	0.18933±0.037	0.23333±0.024*	0.19250±0.021

*$p<0.05$ compared with Mock group and psiRNA –Scramble group; #$p>0.05$ compared with Mock group

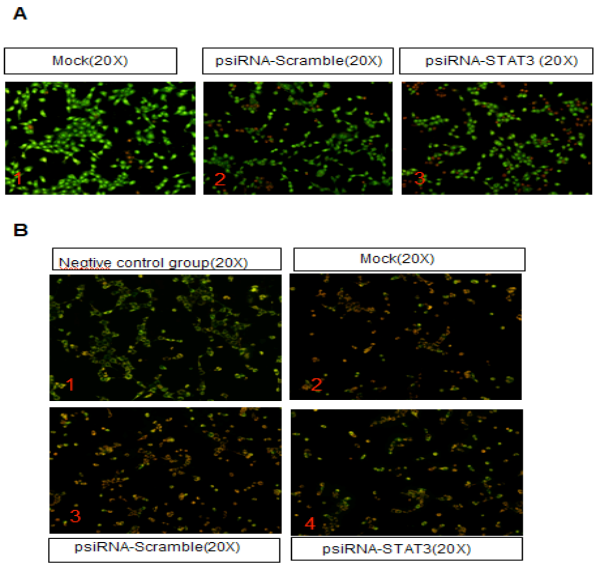

Figure 2. The Effect of Life2000 Transfected psiRNA-STAT3 Plasmid on Apoptosis for 8h

Table 2. Effects of siRNA on Apoptosis (n=5 $\bar{X}$±S)

Group	8h	
	alterations of mitochondrial membrane potential	(AO/EB) double staining
Negtive contro	84.60±8.44**	
Mock	30.40±2.70	24.00±19.506
psiRNA-Scramble	54.40±10.23#	40.20±26.725#
psiRNA -STAT3	72.60±4.33*	73.80±14.653*

*$p<0.05$ Compared with Mock group and psiRNA–Scramble grou; #P>0.05 Compared with Mock group; **$p<0.01$ Compared with Mock group

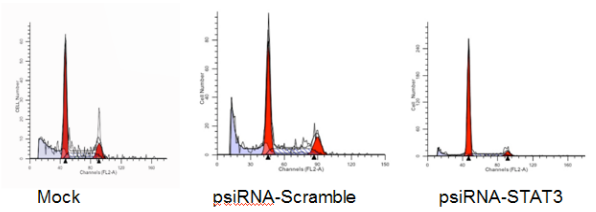

Figure 3. Effect of psiRNA-STAT3 Transfeted with Life2000 for 8h on the Cell Cycle of 4T1 Cells

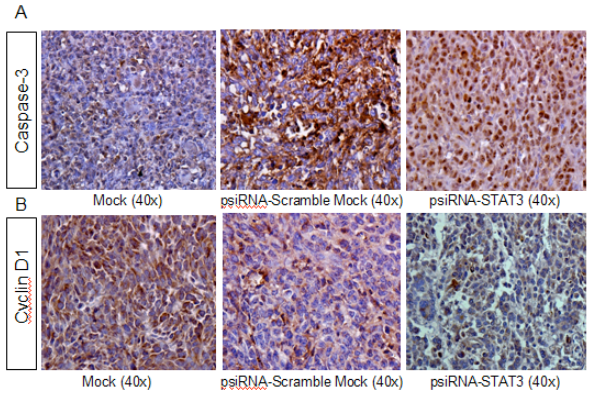

Figure 4. Analysis of Caspase -3 Protein Expression by Immunohistochemistry

Table 3. Effect of siRNA on Cell Cycle (n=3, $\bar{X}$±S)

Group	8h		
	G0/G1	S	G2/M
SMock	45.60+4.44	31.28+6.63	23.12+1072
psiRNA-Scramble	55.37+4.39	28.77+1.25#	15.83+3.32
psiRNA-STAT3	72.08+5.90*	16.91+3.78*	11.13+5.47

*p<0.05 compared with Mock group and psiRNA-Scramble group; #p>0.05 compared with Mock group

4T1 cells at 8 hours in psiRNA-STAT3 group in S phase decreased obviously.psiRNA-STAT3 group has significant difference, compared with Mock group and psiRNA-Scramble group (p<0.05). psiRNA-Scramble group showed no significant difference, compared with Mock group (p>0.05). Three groups showed no significant difference, in G2 and M phase (p>0.05). (Figure 3, Table 3)

Analysis of Caspase -3 protein expression by immunohistochemistry

No expression of Caspase -3 in most tumor cells in Mock group can be observed under the microscope, the cell nucleus were stained light, and there were many nucleolus which were visible obviously, while a few tumor cells expressed Caspase -3;more tumor cells expressed Caspase-3 in psiRNA -Scramble group, the cell nucleus of tumor cells which did not expressed Caspase -3 showed blue, nucleoli were visible; the majority of tumor cells expressed Caspase- 3 in psiRNA-STAT3 group, no expression of Caspase-3 tumor nuclei was blue, and nucleoli were visible. (Figure 4A)

Immunohistochemical analysis of the expression of Cyclin D1 protein

Under the microscope: There were many tumor cells which expressed Cyclin D1 in Mock group, with diffuse distribution; a few tumour cells expressed Cyclin D1 in psiRNA -Scramble group, with scattered distribution ;psiRNA-STAT3 group: the quantities of tumor cells of positive Cyclin D1 was small, and scattered in around. (Figure 4B)

Discussion

Signal transducer and activator of transcription -3 (STAT3) is an important member of the STAT family, currently defined as the oncogene (Chiba et al., 2009). Many tumors overexpress or sustain expressing STAT3 (Renl et al., 2002; Ling et al. 2007; Xue et al., 2012), which indicates that the activation of the STAT3 signaling pathway is closely related to tumorigenesis and development. (Chatter et al., 2000).

In recent years, the gene therapy of breast cancer have developed rapidly.The study of oncogene STAT3 and tumor has become a hot spot, the inhibitor is thought to be a potential anticancer drugs (Shi et al., 2012). Studies have confirmed that, the inhibition to STAT3 by RNA interference can decrease cell proliferation (Madoux et al., 2010). These findings provide an important theoretical basis for STAT3, which become the target for tumor treatment.

This study investigated that the inhibitory effect of Life2000 transfected with psiRAN-STAT3 plasmid on 4T1 tumor cells. We have observed the effect of 6h, 8h, 10h, in different time after transfection on the proliferation of tumor cells. Eight hours after transfection, the number of cells in Mock group increased significantly, the nuclear morphology was normal;the number of cells in psiRNA-Scramble group continued to reduce; cells of the psiRNA-STAT3 group decreased significantly, a large number of round cells emerged in cultured liquid, with floating in the culture fluid. We observed under the fluorescence microscope, using AO/EB double staining, showed that, cells in Mock group were polygonal, with projections, a few early apoptosis cells can be detected occasionally, and late apoptosis cells; in psiRNA-Scramble group, cells were circular, and the early apoptosis and late apoptosis cells increased obviously;The psiRNA-STAT3 group cells changed from polygonal to rounded, and there are many circular and pyknotic apoptotic cells. Results show that, psiRNA-STAT3 group has obvious inhibitory effect on 4T1 breast cancer cells, psiRNA-STAT3 group has significant difference, compared with Mock group, Scramble group (p<0.05). Scramble group showed no significant difference, compared with Mock group (p>0.05). Our results demonstrated that Life2000 transfected psiRNA-STAT3 plasmid after 8 hours can inhibit 4T1 tumor cell proliferation and induce apoptosis.

Current studies show that (Chen et al., 2005), cell cycle regulatory protein CyclinD1, CDK4, Rb are involved in cell cycle conversionof G1 / S phase, and abnormal expression of CDK4 and CyclinD1, the phosphorylation status of Rb protein were related to the occurrence and development of. malignant tumor. As a cell cycle regulatory protein Cyclin D1, which activated CDK4, mediated the phosphorylation of Rb protein so that the cells skip over the control point, then enter the S

phase, resulting in cell proliferation and tumorigenesis. There were many tumor cells which expressed Cyclin D1 in Mock group, with diffuse distribution; a few tumour cells expressed Cyclin D1 in psiRNA -Scramble group, with scattered distribution ;in psiRNA -STAT3 group:the quantities of tumor cells of positive Cyclin D1 were small, and scattered in around. Cell cycle analysis showed that, at 8h after transfection, the number of cells in psiRNA-STAT3 group increased significantly in D0/D1 phase, the number of cells decreased obviously in S phase, with significant difference, compared with the Mock group and psiRNA-Scramble group $p<0.05$. The mechanism may be that, psiRNA-STAT3 downregulated the expression of the target genes CyclinD1 protein and had strong inhibition effect on cells which enter into the S phase from the G0/G1 phase, thereby inhibiting the synthesis of cellular DNA.

Apoptosis mainly has the death receptor pathway and mitochondrial pathway, Caspase-3 is the end effector molecules (Hishikawa et al., 2000; Kaufmann et al., 2008) of these two approaches. Mitochondria play an important role in apoptosis, and mitochondrial membrane potential decrease is considered a sign of early apoptosis (Grebenová et al., 2003). The damage of the integrity and permeability of mitochondrial membrane caused that, the mitochondrial membrane potential disappeared and released a variety of apoptosis inducing factor such as cytochrome C, procaspase-3, procaspase-8, and procaspase-9 (Desagher et al., 2000). Caspse-3 as a downstream signal molecule in apoptosis pathway, can be a direct response to apoptosis of tumor cells (Kollmar et al., 2010). The process occurs in the early apoptosis.This research is based on the JC-1 detection of the influence of psiRNA-STAT3 plasmid on 4T1 cell mitochondrial membrane potential.The results showed that, 8 hours after transfection, the mitochondrial transmembrane potential in psiRNA-STAT3 group was decreased obviously, compared with the Mock group and psiRNA-Scramble group with a significant difference $p<0.05$, Mock group showed no significant difference, compared with psiRNA-Scramble group $p>0.05$. Research shows that the psiRNA-STAT3 plasmid can induce the apoptosis of 4T1 tumor cells, and dominated in the early apoptosis. This study was consistent with the cell cycle and results of AO/EB double staining . Meanwhile, the immunohistochemistry showed that most of the tumor cells in Mock group did not express Caspase -3, the cell nucleus was stainined light, nucleolus was obvious;in psiRNA-Scramble group, more tumor cells expressed Caspase and -3, and clear nucleolus;the majority of tumor cells did not express Caspase- 3 in psiRNA-STAT3 group, no expression of Caspase- 3 tumor nuclei was blue, and nucleoli were visible.This study demonstrated that by blocking Caspse-3 protein expression, apoptosis pathway is activated, thus plays the anti- tumor effect.

In summary, Life2000 transfected psiRNA -STAT3 plasmid could obviously inhibit the proliferation of mouse breast cancer 4T1 cells and induce apoptosis. The molecular mechanism of the apoptosis is not only associated with the mitochondrial pathway of apoptosis, but also closely related with the death receptor pathway and the expression CyclinD1 protein. Therefore, strengthening the research on the pathogenesis of expression of cell signal transduction pathway and cell cycle protein helps to elucidate the pathogenesis of breast cancer so that provide a theoretical basis for the clinical treatment of breast cancer.

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