Recombinant *Escherichia coli* Trx-JZTX-III represses the proliferation of mouse hepatocellular carcinoma cells through induction of cell cycle arrest

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Abstract. The aim of the present study was to investigate the effects of recombinant *Escherichia coli* (*E. coli*) Trx-jingzhaotoxin (JZTX)-III on cell growth in the mouse hepatocellular carcinoma (HCC) cell line Hepa1-6. The *JZTX-III* gene sequence was synthesized and cloned into the pET-32a(+) vector to construct the recombinant fusion protein Trx-JZTX-III, which was subsequently purified. Hepa1-6 cells were treated with 0 to 1,000-μg/ml concentrations of Trx-JZTX-III; this was demonstrated to affect cell viability, as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay. The expression of the proliferating cell nuclear antigen (PCNA) protein was investigated using western blot analysis. A colony formation assay was used to determine Hepa1-6 cell proliferation, and the migration ability of cells was determined using a wound-healing assay. Additionally, flow cytometry was employed to observe changes in the cell cycle. The MTT assay and quantification of PCNA expression indicated that recombinant *E. coli* Trx-JZTX-III significantly repressed the proliferation of Hepa1-6 cells. Colony formation and the migration of malignant cells was inhibited following treatment with recombinant *E. coli* Trx-JZTX-III. Flow cytometry showed that recombinant *E. coli* Trx-JZTX-III induced G_0/G_1 cell cycle arrest. In conclusion, recombinant *E. coli* Trx-JZTX-III functions as a tumor suppressor drug in mouse HCC and its underlying mechanism may involve the induction of G_0/G_1 cell cycle arrest.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and the third most common cause of cancer-related mortality worldwide (1). The treatment of HCC is currently challenging and may remain difficult in the future (2). Although a few novel methods have been applied (3), pharmacotherapy remains the routine treatment for HCC.

Venom from natural toxins has been considered for development into anticancer drugs (4) and the value of their potential biomedical application is promising. Animal toxins have also been identified to have antitumor activity, including bee (5), snake (6) and scorpion venom (7). Recently, scorpion venom was shown to induce cell apoptosis (8) and snake toxin was demonstrated to inhibit the proliferation of PA-1 and SK-OV3 cells (human ovarian cancer cells) (9).

Spider venom isolated from *Lycosa singorensis* has exhibited an ability to inhibit cancer cell growth *in vitro* (10). Spider venom is a liquid with offensive, defensive and digestive functions. Furthermore, different species of spiders vary in toxicity; homology is limited even in primary structures. There are a large number of spider species in China; *Chilobrachy jingzhao* is one of these and is mainly located in the Guangxi Province of China, with the following classification: Phylum, Arthropoda; class, Arachnids; order, Araneae; family, Theraphosidae. *Chilobrachy* jingzhaotoxin (JZTX)-III is a common biological resource that may play a role in directly killing tumor cells. Therefore, JZTX-III has the potential to be used in the development of anticancer drugs and has received increasing attention from pharmaceutical scientists and molecular biologists.

The present study aimed to investigate the potential use of recombinant *Escherichia coli* (*E. coli*) Trx-JZTX-III in the treatment of HCC by examining the proliferation of Hepa1-6 cells, a HCC cell line from mice, following treatment with or without recombinant *E. coli* Trx-JZTX-III.

Materials and methods

Preparation of recombinant E. coli Trx-JZTX-III. In this study, genetic engineering was utilized to inhibit the tetrodotoxin-resistant sodium channel. JZTX-III was inserted in the prokaryotic expression vector pET-32a(+) to create a recombination plasmid which expressed the fusion protein Trx-JZTX-III in *E. coli*, using Trx as a protein tag. The recombinant *E. coli* Trx-JZTX-III was freeze-dried and stored at -80˚C until use. The study was approved by the ethics committee of Harbin Medical University, Harbin, China.

Cell culture. The HCC cell line Hepa1-6 was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai Branch Cell Bank, Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) containing 10% (v/v) fetal bovine serum (FBS; Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2.

MTT assay. The growth and viability of Hepa1-6 cells were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT; Sigma, St. Louis, MO, USA) assay. The cells were plated into 96-well microtiter plates at a density of $5x10^3$ cells/well. Following incubation for 24 h, recombinant *E. coli* Trx-JZTX-III was serially diluted to various concentrations (1,000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 μg/ml) and added to each well. A control group was treated with phosphate-buffered saline (PBS; Gibco) alone. After incubation for 24 and 48 h, 200 μl MTT (5 mg/ml) solution was added to each well and cultured for 4 h at 37˚C. Formazan crystals were solubilized using 150 μl DMSO (Sigma) and plates were agitated for 10 min. The absorbance was measured at 490 nm and a colorimetric MTT assay was performed to investigate cell growth. All the experiments were repeated three times.

Western blot analysis. Hepa1-6 cells ($5x10^5$) were seeded in 6-well plates and treated with 0, 600, 800 and 1,000 μg/ml recombinant *E. coli* Trx-JZTX-III for 48 h, and proteins were then extracted from each group of cells. The proteins were separated by 10% SDS-PAGE and subsequently transferred to PVDF membranes. Nonfat milk (5%) in TBST was used to block the membranes at room temperature for 2 h. The membranes were then incubated with rabbit anti-proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or mouse anti-β-actin antibodies (Santa Cruz Biotechnology, Inc.) at 4˚C overnight. Next, the membranes were incubated with anti-rabbit or anti-mouse secondary antibodies for 2 h. Finally, the membranes were washed three times with TBST and exposed to X-ray film.

Colony formation assay. A colony formation assay was conducted by plating Hepa1-6 cells into 6-well plates (200 cells/plate). Following a 24-h incubation, recombinant *E. coli* Trx-JZTX-III at various concentrations (600, 800 and 1,000 μg/ml) was added; the control group received no recombinant *E. coli* Trx-JZTX-III. After 2 weeks, the cells were stained with Giemsa. Colonies were counted only when a single clone contained >50 cells.

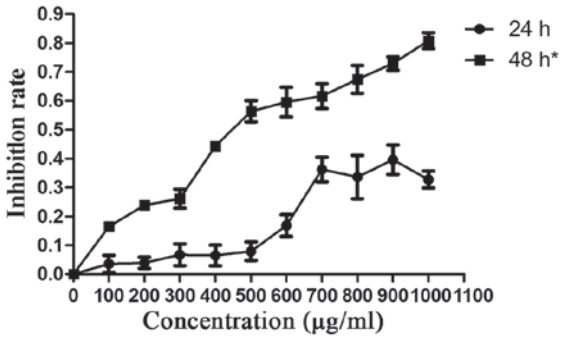

Figure 1. Effects of recombinant *E. coli* Trx-JZTX-III on Hepa1-6 cells for 24 and 48 h, as determined by the MTT assay. After a 48-h incubation, increased inhibition rates were observed with higher concentrations of recombinant *E. coli* Trx-JZTX-III, indicating that the activity of Hepa1-6 cells was dose-dependent. The results are expressed as a percentage of the control. *P<0.05. *E. coli*, *Escherichia coli*; JZTX-III, jingzhaotoxin-III; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide.

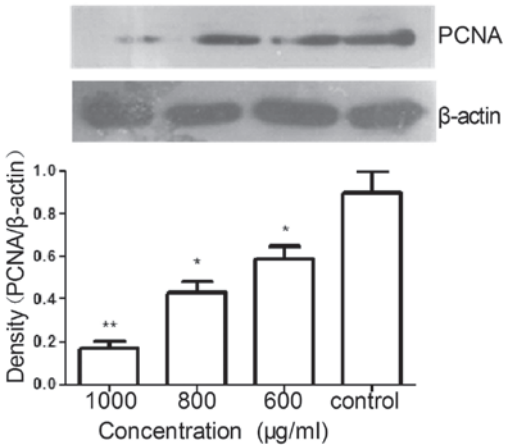

Figure 2. Effect of recombinant *E. coli* Trx-JZTX-III on proliferating cell nuclear antigen (PCNA) protein expression in Hepa1-6 cells, as determined by western blot analysis. The expression of PCNA protein was detected in each group. The level of PCNA protein expression was downregulated with 600, 800 and 1,000 μg/ml *E. coli* Trx-JZTX-III compared with the control group (**P<0.01; *P<0.05). *E. coli*, *Escherichia coli*; JZTX-III, jingzhaotoxin-III.

Cell migration assay. Cell migration was determined using a wound-healing assay. Hepa1-6 cells were seeded at a density of $5x10^5$ cells/6-well plate in DMEM containing 10% FBS. A scratch wound was created on the confluent cell monolayer using a 200-μl pipette tip, and then the cells were treated with various concentrations of recombinant *E. coli* Trx-JZTX-III (0, 600, 800 and 1,000 μg/ml). The wounded areas were observed and images were captured using a microscope at 0 and 48 h after scraping.

Flow cytometry. Hepa1-6 cells were seeded into 6-well plates in DMEM containing 10% FBS and treated with various concentrations of recombinant *E. coli* Trx-JZTX-III (0, 600, 800 and 1,000 μg/m) for 48 h. The cells were harvested, washed in cold PBS and then fixed in 75% alcohol at 4˚C for ≥12 h. The fixed cells were resuspended in PBS containing 50 g/l RNase A and 50 mg/l propidium iodide (PI) for 30 min, and analyzed using flow cytometry (Becton Dickinson, USA).

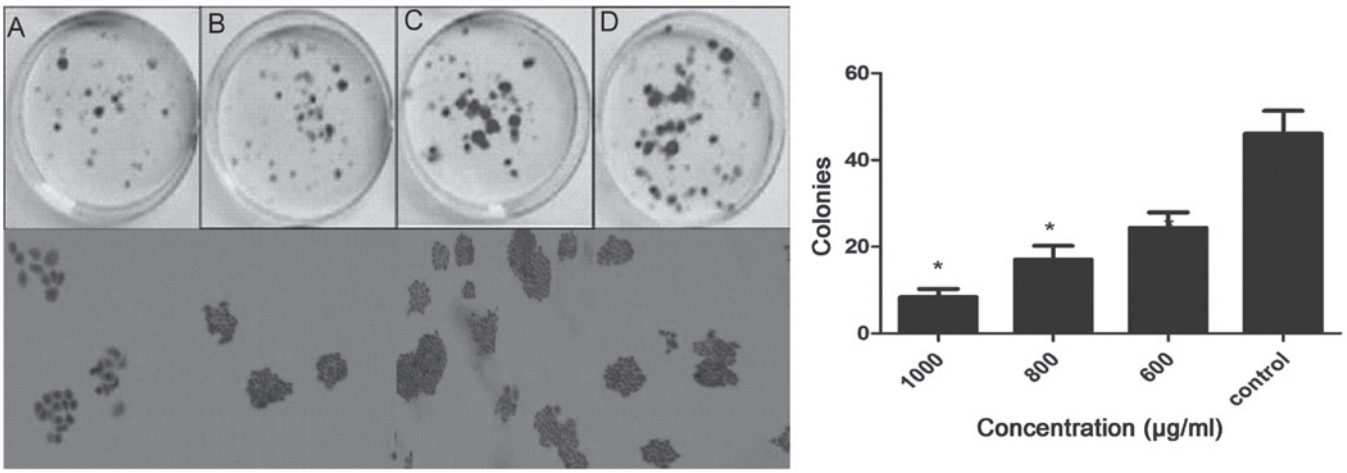

Figure 3. Colony formation of Hepa1-6 cells following treatment with (A) 1,000, (B) 800, (C) 600 and (D) 0 μg/ml recombinant *E. coli* Trx-JZTX-III. The colony formation ability of Hepa1-6 cells was significantly inhibited by *E. coli* Trx-JzTx-III in a dose-dependent manner when compared with the control group (*P<0.05). *E. coli*, *Escherichia coli*; JZTX-III, jingzhaotoxin-III.

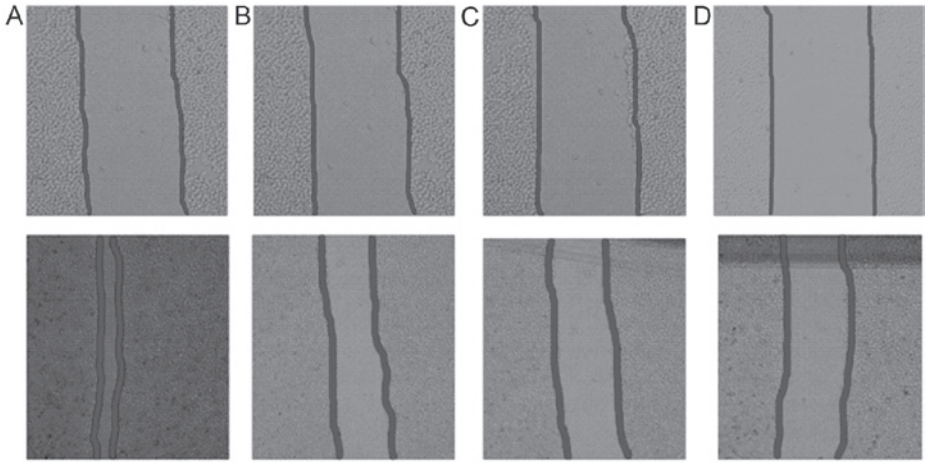

Figure 4. The effect of (A) 0, (B) 600, (C) 800 and (D) 1,000 μg/ml *E. coli* Trx-JZTX-III on a wounded area was investigated. In the migration assay, our results demonstrate that the migration was significantly decreased when the cells were treated with recombinant E.coli Trx-JZTX-III (600, 800 and 1,000 μg/ml). *E. coli*, *Escherichia coli*; JZTX-III, jingzhaotoxin-III.

Statistical analysis. The data are presented as the mean ± SEM. P<0.05 was considered to indicate a statistically significant difference. The results were analyzed by Student's t-test using SPSS version 10.0 (SPSS, Inc., Chicago, IL, USA).

Results

Recombinant E. coli Trx-JZTX-III inhibits Hepa1-6 cell proliferation. Hepa1-6 cells were exposed to various concentrations of *E. coli* Trx-JZTX-III (1,000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 μg/ml). Growth inhibition was most significant at a treatment time of >48 h (Fig. 1). Therefore, we further investigated the *in vitro* inhibitory effects of recombinant *E. coli* Trx-JZTX-III using a treatment time of 48 h (P<0.05).

In order to obtain additional evidence, western blot analysis was performed; PCNA was used as a reporter for proliferation. Hepa1-6 cells were treated with recombinant *E. coli* Trx-JZTX-III for 48 h and the expression of PCNA protein was determined using western blot analysis. The expression of PCNA was significantly lower with 600, 800 and 1,000 μg/ml *E. coli* Trx-JZTX-III treatment compared with cells in the control group (Fig. 2; P<0.05).

Recombinant E. coli Trx-JZTX-III represses colony formation and cell migration. A colony formation assay was used to assess the colony-forming ability of Hepa1-6 cells following treatment with or without recombinant *E. coli* Trx-JZTX-III. Compared with cells in the control group (Fig. 3D), cells treated with 600 (Fig. 3C), 800 (Fig. 3B) and 1,000 μg/ml (Fig. 3A) recombinant *E. coli* Trx-JZTX-III formed fewer and smaller colonies.

Compared with the control group (Fig. 4A), a significant decrease in Hepa1-6 cell migration was treated with recombinant *E.coli* Trx-JZTX-III (Fig. 4B-D).

Recombinant E. coli Trx-JZTX-III induces Hepa1-6 G_0/G_1 cell cycle arrest. As shown in Fig. 5, cells treated with recombinant *E. coli* Trx-JZTX-III at concentrations of 600 (Fig. 5C),

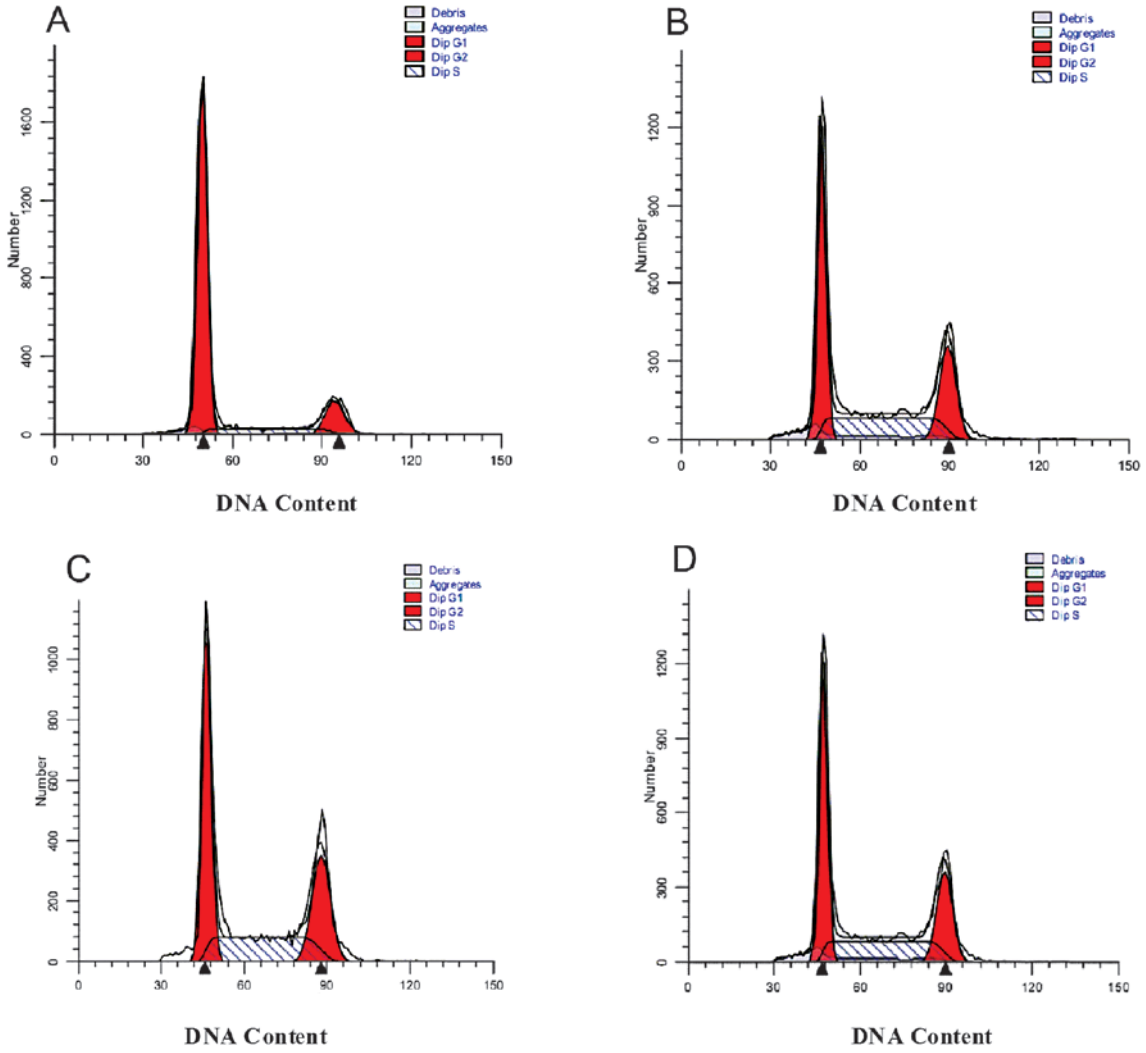

Figure 5. Flow cytometric analysis of each group of Hepa1-6 cells. Exposure of Hepa1-6 cells to (A) 1,000, (B) 800 and (C) 600 mg/l *E. coli* Trx-JZTX-III for 48 h significantly increased the cell population in the S and G_2/M phases. (D) The control group. *E. coli*, *Escherichia coli*; JZTX-III, jingzhaotoxin-III.

Table I. Cell cycle distribution of Hepa1-6 cells (mean ± SEM).

	Cell cycle distribution (%)		
Group	G_0/G_1[a]	S[a]	G_2/M[a]
Trx-JZTX-III (μg/ml)			
1000	72.2±3.5	13.96±1.9	28.4±1.3
800	45.7±2.0	29.45±3.7	54.3±2.4
600	42.1±2.9	31.18±4.2	57.9±2.0
Control	40.8±1.9	35.10±1.4	59.24±1.2

[a]P<0.05 compared with the control group, indicating a significant increase. JZTX-III, jingzhaotoxin-III.

800 (Fig. 5B) or 1,000 μg/ml (Fig. 5A) exhibited a significantly increased cell population in the G_0/G_1 phase when compared with the control group (Fig. 5D). However, cells treated with recombinant *E. coli* Trx-JZTX-III demonstrated a significantly decreased cell population in the G_2/M or S phases (P<0.01; Table I).

Discussion

Currently, several types of JZTXs are derived from the spider *Chilobrachys jingzhao*, including JZTX-II (11), -IX (12), -V (13), -XI (14), -IV (15) and -XIII (16). JZTX-III (molecular weight, 3919.3 Da) is a peptide toxin, containing 36 residues and three pairs of intracellular disulfide bridges (I-IV, II-V, and III-VI) (17).

The venom from this spider has been shown to induce apoptosis in the myelogenous leukemia K562 cell line (18) and in MCF-7 cells (19). Spider venom has been demonstrated to be a novel tumor suppressor drug in HeLa cells (20) and was also shown to inhibit the proliferation of HepG2 cells (21) and induce BEL-7402 cell apoptosis (22) in HCC.

Spider venom is usually obtained by direct extraction and artificial chemical synthesis. In this study, our aim was to obtain a significant amount of recombinant fusion protein of Trx-JZTX-III. Compared with natural JXTX-III, recombinant *E. coli* Trx-JZTX-III has advantages with regard to mass production and cost efficiency.

The MTT assay and quantification of PCNA expression indicated that recombinant *E. coli* Trx-JZTX-III significantly

repressed the proliferation of Hepa1-6 cells. Furthermore, the colony formation ability and migration of the malignant cells were inhibited following treatment with recombinant *E. coli* Trx-JZTX-III. Thus, recombinant *E. coli* Trx-JZTX-III functioned as a tumor suppressor drug.

To explain the underlying mechanism of recombinant *E. coli* Trx-JZTX-III in suppressing Hepa1-6 cell proliferation, we investigated the cell cycle of Hepa1-6 cells following treatment with or without recombinant *E. coli* Trx-JZTX-III. Recombinant *E. coli* Trx-JZTX-III was shown to induce G_0/G_1 cell cycle arrest; this may be one of the mechanisms that mediate the antitumor function of recombinant *E. coli* Trx-JZTX-III.

The present study demonstrated that recombinant *E. coli* Trx-JZTX-III functioned as a tumor suppressor drug in mouse HCC cell proliferation, potentially via the induction of G_0/G_1 cell cycle arrest. These results suggest that the antitumor activity of recombinant *E. coli* Trx-JZTX-III may be associated with the proliferation of the mouse cell line Hepa1-6. However, the effect of recombinant *E. coli* Trx-JZTX-III on tumors *in vivo* remains unknown. Future investigation with regard to the association between recombinant *E. coli* Trx-JZTX-III and human hepatoma cells is required to identify the specific molecules which are involved in the antitumor activity and synthesis of recombinant *E. coli* Trx-JZTX-III. Results of the present study suggest that recombinant *E. coli* Trx-JZTX-III may be a promising candidate for further development as a therapeutic agent due to its anticancer efficacy. In conclusion, recombinant *E. coli* Trx-JZTX-III represents a potentially important tool for the development of novel drugs and antitumor agents in the treatment of HCC.

Acknowledgements

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