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ORIGINAL ARTICLE

# *In vitro* study of the effect of small interfering ribonucleic acid on the expression of FOXN1 and B cell-attracting chemokine 1 in thymoma cell lines

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BCA1; FOXN1; RNA interference; thymoma cell lines.

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## Abstract

**Background:** To determine the relationship between FOXN1 (a transcription factor) and B cell-attracting chemokine 1 (BCA1, a chemotactic factor), and their influence on thymoma cell proliferation.

**Methods:** We initially used immunohistochemical methods to compare the expression levels of FOXN1 and BCA1 in thymoma and non-thymomatous tissue samples. Reverse transcription polymerase chain reaction (RT-PCR) and Western blotting were used to compare the expression of FOXN1 and BCA1 in thymoma cells (Thy0517) and normal thymic epithelial cells (CRL7660). We used ribonucleic acid interference (RNAi) to downregulate FOXN1 and BCA1 expression in Thy0517 cells to determine the relationship of the two factors with cell regulation. We also performed methyl thiazolyl tetrazolium (MTT) [3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide] assays to detect the changes in Thy0517 cells after RNAi of FOXN1 and BCA1.

**Results:** FOXN1 and BCA1 expression levels were higher in thymoma tissues and Thy0517 cells compared to non-thymomatous tissue and CRL7660 cells ($P < 0.05$). RT-PCR and Western blot following RNAi showed that FOXN1 controlled BCA1 expression. MTT assay showed that FOXN1 and BCA1 downregulation rapidly inhibited Thy0517 cell proliferation.

**Conclusions:** FOXN1 and BCA1 expression was higher in thymoma tissue samples and cell lines than in non-thymomatous tissue and normal thymic epithelial cells. FOXN1 acts upstream of BCA1 and both FOXN1 and BCA1 promote thymoma cell proliferation.

## Introduction

The transcriptional factor, FOXN1, and the chemotactic factor, B cell-attracting chemokine 1 (BCA1), are recently discovered cytokines relating to thymoma and myasthenia gravis (MG). FOXN1, which is mainly expressed in thymic epithelial cells (TECs) and belongs to the FOX family, plays an important role in the development of T cells in the thymus, where the expression of FOXN1 in thymoma is higher than in normal thymic tissue.[1–5] BCA1 is overexpressed in many autoimmune diseases and tumors where BCA1 is involved in the process of proliferation and migration of tumor cells.[6–10] The results of immunohistochemical studies showed that the expression of FOXN1 and BCA1 was higher in thymoma tissue samples than in non-thymomatous tissue samples. Ribonucleic acid interference (RNAi) was used to determine the relationship between FOXN1 and BCA1, and the influence of both factors on the proliferative behavior of thymoma cells.

## Materials and methods

### Sample collection and immunohistochemical staining

A total of 88 cases of thymic disease were collected from the Tianjin Medical University Affiliated General Hospital, Tianjin, China. Thymic tissue specimens were collected from patients who underwent thymectomy from 2001 to 2011. The patients were divided into four groups: thymoma; myasthenia gravis combined with thymoma (MG/thymoma); myasthenia gravis combined with thymic lymphoid follicular

**Table 1** Demographic data of cases

| | | Control group | MG/LFH group | MG/thymoma group | Thymoma group | *P*-value |
|---|---|---|---|---|---|---|
| Gender | Male | 6 | 6 | 25 | 5 | 0.858† |
| | Female | 9 | 6 | 24 | 7 | |
| Age (years) | | 18–71 | 19–64 | 27–74 | 33–75 | |
| | Mean ± SD | 51.87 ± 15.46 | 48.08 ± 18.38 | 49.92 ± 11.91 | 55.92 ± 11.09 | 0.366‡ |
| MG subtype | Mild | | 8 | 20 | | 0.110† |
| | Intermediate-Severe | | 4 | 29 | | |
| Thymoma subtype | A | | | 4 | 3 | 0.146† |
| | AB | | | 10 | 4 | |
| | B1 | | | 11 | 1 | |
| | B2 | | | 8 | 0 | |
| | B3 | | | 3 | 2 | |
| | Mixed type | | | 13 | 2 | |
| Masaoka stage | I | | | 13 | 3 | 0.958† |
| | II | | | 30 | 8 | |
| | III | | | 4 | 0 | |
| | IVa | | | 2 | 1 | |
| | IVb | | | 0 | 0 | |

†Mann-Whitney U test. ‡t-test. LFH, lymphoid follicular hyperplasia; MG, myasthenia gravis.

hyperplasia (MG/LFH); and the control group. The control group, consisting of individuals that had been referred to the hospital for mediastinal masses, included five cases of bronchial cysts, three cases of benign teratoma, four cases of thymic cysts, two cases of pericardial cysts, and one case of mesothelial cyst, according to pathology results. The demographic data of all cases are shown in Table 1.

The thymoma histological subtypes were classified according to the World Health Organization classification of thymic epithelial tumors.[11] The thymomas were staged according to the Masaoka staging system.[12] The subtypes of MG were classified according to the clinical classification of the Myasthenia Gravis Foundation of America.[13] Informed consent was obtained from each participant according to protocols approved by the institutional review board of the Tianjin Medical University Affiliated General Hospital, Tianjin, China.

Tissues obtained from thymectomy were fixed with 10% formalin, embedded in paraffin and cut into 4-μm sections for immunohistochemical staining. Samples were de-waxed, re-hydrated through stepwise handling with graded ethanol treatments, and washed in double-distilled water. Antigen retrieval was carried out in 10 mM sodium citrate buffer, pH 6.0 at 95–100°C for 20 minutes before staining. Endogenous peroxidase activity was quenched by 3% hydrogen-peroxide-methanol solution. Sections were incubated with antibodies directed against FOXN1 (1:50; Abcam, Cambridge, MA, UK) and BCA1 (1:100; R&D, Minneapolis, MN, USA) in phosphate buffered saline (PBS) at 4°C overnight. Endogenous peroxidase activity was blocked with hydrogen peroxide. FOXN1 and BCA1 were detected using a biotinylated horse antigoat antibody (1:100; Vector Laboratories, Burlingame, CA, USA) and incubated for 30 minutes. After the application of the secondary antibody, streptavidin-horseradish-peroxidase conjugate was applied. The complex was visualized with 3,3 diaminobenzidene and enhanced with copper sulfate. The slides were washed in distilled water, counterstained with hematoxylin, dehydrated, and mounted with permanent media. Appropriate positive and negative controls were included with the study section. The extent of staining was estimated and graded as follows: 1+, <25%; 2+, 25% to <50%; 3+, 50% to 75%; 4+, ≥75%.

## Cell lines and culture

The CRL7660 cell line (American Type Culture Collection) was established from normal thymic tissue obtained from a patient who had metastatic fibrosarcoma. The Thy0517 cell line (provided by the Department of Cardiac and Thoracic Surgery, Tianjin Medical University Affiliated General Hospital, Tianjin, China) was derived from Type AB thymoma tissue from a thymoma patient. The cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (FBS) to a final concentration of 10% and maintained in a cell culture incubator at 37°C in a humidified atmosphere with 5% $CO_2$.

## Treatment of cells with small interfering ribonucleic acid against FOXN1 and B cell-attracting chemokine 1 (BCA1)

Thy0517 cells were seeded into a six-well plate, at $2 \times 10^5$ cells per well, in an antibiotic-free normal-growth medium supplemented with 10% FBS, and incubated until 70%

  

confluency was achieved. The cells were then transfected with 4 μg each of FOXN1 small interfering RNA (siRNA), BCA1 siRNA and control siRNA duplex, for six hours following the manufacturer's protocol (GeneCopoeia, Rockville, MD, USA) using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). The growth medium was then replaced, and the cells were incubated for an additional 24 hours for reverse transcription polymerase chain reaction (RT-PCR) or for 72 hours for Western blot assay. The efficacies of FOXN1 and BCA1 silencing were determined by RT-PCR and Western blot analysis, respectively. Chemically modified Stealth siRNA targeting FOXN1 (sequence: GCTTCAGCTGCTCGTCATT), BCA1 (sequence: CCTGATGCTGATATTTCCA), and control siRNA were purchased from GeneCopoeia.

## Reverse transcription polymerase chain reaction

Total cellular RNA was extracted using an RNeasy total RNA isolation kit (TianGen, Beijing, China). We used 1 μg total RNA isolated from FOXN1- and BCA1-silenced Thy0517 cells to synthesize cDNA by using the Moloney murine leukemia virus reverse transcriptase enzyme according to the manufacturer's protocol (Takara, Dalian, China). Subsequently, the TS-FOXN1 and BCA1 products were amplified by PCR with a hot start at 94°C for three minutes; 35 cycles each of denaturation at 94°C for three seconds, annealing at 56°C for 30 seconds and elongation at 72°C for 30 seconds; followed by a final step at 72°C for 10 minutes (Bio-Rad, Hercules, CA, USA). The cDNA of FOXN1 and BCA1 were amplified in 25 μl of reaction mixture containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 μm of each primer, and 0.625 U Taq DNA polymerase (TransGen, Beijing, China). In addition, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as a control and amplified in parallel with each sample. The primer sequences for FOXN1, BCA1, and GAPDH were designed using Primer-BLAST and DNAstar software (primer sequences are listed in Table 2). Each PCR included a negative control with $ddH_2O$ instead of cDNA. After PCR, 5 μl of the amplified products were resolved in 1.5% ethidium bromide–stained agarose gels, visualized in ultraviolet light and photographed. Volumetric integration of signal intensities was performed using NIH Image software. The relative levels of FOXN1 and BCA1 messenger (m)RNA expression were determined based on their individual signal density to GAPDH ratio.

**Table 2** Sequences of RT-PCR primers for FOXN1, BCA1 and GAPDH

| Name | Primer Sequences |
|---|---|
| FOXN1 5′-primer | AGAGTGGTGCTGGGATGTT |
| FOXN1 3′-primer | GATGCTATAGGAATAGATGGGT |
| BCA1 5′-primer | AAACTCACTACGGA GGAG |
| BCA1 3′-primer | ATTAGATACCAGAGTCCC |
| GAPDH 5′-primer | GAAGGTGAAGGTCGGAGTC |
| GAPDH 3′-primer | GGGTGGAATCATATTG |

BCA1, B cell-attracting chemokine 1; GADPH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription polymerase chain reaction assays.

## Immunoblotting and antibodies

Immunoblot analysis was conducted on untreated total cell lysates or lysates from Thy0517 cells treated with FOXN1 siRNA, BCA1 siRNA or control siRNA. Cells were lysed in a buffer containing 50 mM Tris-hydrochloride (HCl) at pH 7.4, 150 mM NaCl and 1% NP-40 supplemented with protease and phosphatase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). The protein concentration of the cell lysates was determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Equal amounts (50 μg) of cell lysates were denatured by boiling in Laemmli buffer for 10 minutes, resolved on 5%–12% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes using a semi-dry transfer cell system (Bio-Rad, Hercules, CA, USA). The membranes were then blocked for one hour at room temperature in 5% non-fat milk in 1 × Tris-Tween–buffered saline (TBST; 30 mM Tris-Base, 150 mM NaCl, and 0.1% Tween 20), followed by washing with 1 × TBST. Primary antibodies against FOXN1 (Abcam, 1:1000), BCA1 (Abcam, 1:1000) or β-actin (Abcam, 1:1000) were added to the membrane and incubated for 16 hours at 4°C in 1 × TBST. The membranes were then washed and the corresponding horseradish peroxidase–conjugated secondary antibodies were added for one hour, followed by additional washes. Immunoreactive proteins were visualized on autoradiographic films using a chemiluminescent detection reagent (Pierce). The protein levels of FOXN1 and BCA1 were normalized with that of β-actin.

## Methyl thiazolyl tetrazolium cell proliferation assay

To assess the effect of FOXN1 and BCA1 on thymoma cell growth and viability, Thy0517 cells were treated with FOXN1 siRNA, BCA1 siRNA and control siRNA, and their proliferation was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, treated cells were seeded in 96-well plates at a density of 5000 cells per well per 100 μl media and allowed to adhere for six hours. Plates in triplicates were then incubated for 24, 48, and 72 hours. Each day, three hours prior to end of the incubation time, the plates were centrifuged at 200 *g* for five minutes to pellet potential floating cells. The media were then aspirated out of the wells, and 100 μl fresh media containing 10%

 

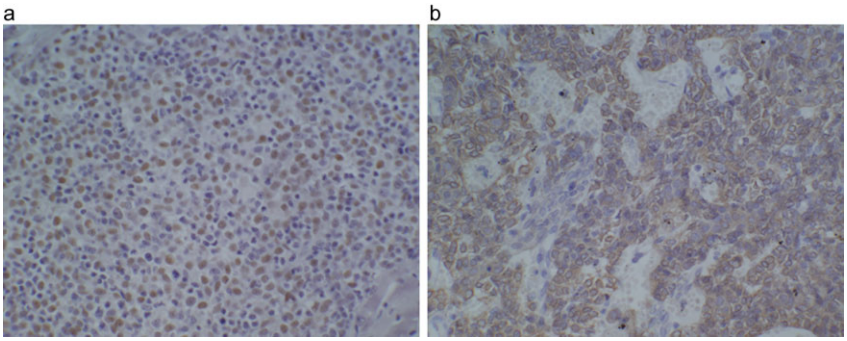


**Figure 1** Immunohistochemical staining of FOXN1 and B cell-attracting chemokine 1 (BCA1) in thymoma tissues. (**a**) FOXN1 staining in thymoma tissue; (**b**) BCA1 staining in thymoma tissue. Original magnification 200×.

volume/volume MTT was added to each well. Plates were returned to the incubator for an additional three hours. At the end of the incubation time, media were aspirated out of the wells, and 200 μl dimethyl sulfoxide was added to each well to solubilize the formazan crystals. Plates were then read using a microplate spectrophotometer at 540 nm. The absorbance of formazan dye solution was directly proportional to the number of proliferating cells per cell.

### Statistical analysis

RT-PCR, Western blot, and MTT assays were performed in triplicate, and at least three separate studies with similar results were performed using unpaired *t*-tests, as appropriate. Statistical analyses were performed using the SPSS system v.19.0. A *P*-value of <0.05 was considered statistically significant.

## Results

### Immunohistochemical features

FOXN1 was diffusely expressed with a strong nuclear staining pattern in all cases of thymoma that were type B, AB, B2, B3 and mixed (Fig 1a); 12 cases of type AB were negative. The positive rates of FOXN1 in the MG/thymoma (38/49, 77.55%) and thymoma groups (11/12, 91.67%) were higher than in the control (2/15, 13.33%) and MG/LFH groups (5/12, 41.67%) (Table 3).

BCA1 was diffusely expressed with a cytoplasmic staining pattern in predominantly epithelial cells in lymphoid hyperplasia and thymoma tissue (Figs 1b, 3), and other BCA1-positive cell types included lymphoid and dendritic cells. The positive rates of BCA1 in the MG/thymoma (49/49, 100%) and thymoma groups (12/12, 100%) were higher than in the control (3/15, 23.08%) and MG/LFH groups (8/12, 66.67%) (Table 3).

### Expression of FOXN1 and BCA1 in thymoma and normal thymic epithelial cells

After the amplifications of cDNA, the expression of both FOXN1 mRNA and BCA1 mRNA was significantly higher in the Thy0517 cells than in the CRL7660 cells ($P = 0.000$; Figs 2, 3). The levels of FOXN1 mRNA and BCA1 mRNA were $1.30 \pm 0.49$ and $0.69 \pm 0.76$, respectively, in Thy0517 cells and $1.66 \pm 0.31$ and $0.41 \pm 0.01$, respectively, in CRL7660 cells.

Western blot results also showed that the expression of both FOXN1 and BCA1 were significantly higher in Thy0517 cells than in CRL7660 cells ($P = 0.000$; Fig 4). The levels of

**Table 3** The expression levels of FOXN1 and BCA1 in different types of thymus tissue (cases)

| | FOXN1 | | | BCA1 | | |
|---|---|---|---|---|---|---|
| | + | – | *P* value* | + | – | *P* value* |
| Control group | 2 | 13 | | 3 | 12 | |
| MG/LFH group | 5 | 7 | 0.101 | 8 | 4 | 0.016 |
| MG/thymoma group | 38 | 11 | 0.000 | 49 | 0 | 0.000 |
| Thymoma group | 11 | 1 | 0.000 | 12 | 0 | 0.000 |

*Compared with control group, Mann-Whitney U test. BCA1, B cell-attracting chemokine 1; LFH, lymphoid follicular hyperplasia; MG, myasthenia gravis.

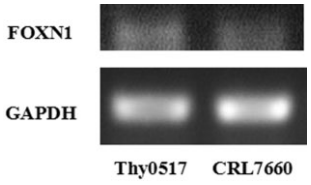


**Figure 2** Messenger ribonucleic acid expression of FOXN1 in Thy0517 and CRL7660 cells according to reverse transcription polymerase chain reaction assays. GADPH, glyceraldehyde 3-phosphate dehydrogenase.

  

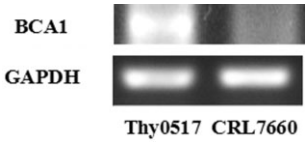


**Figure 3** Messenger ribonucleic acid expression of B cell-attracting chemokine 1 (BCA1) in Thy0517 and CRL7660 cells, determined using reverse transcription polymerase chain reaction assays. GADPH, glyceraldehyde 3-phosphate dehydrogenase.

FOXN1 and BCA1 proteins were 0.72 ± 0.12 and 0.37 ± 0.03, respectively, in Thy0517 cells and 0.8 ± 0.002 and 0.42 ± 0.03, respectively in CRL7660 cells.

## Inhibition of FOXN1 lowers expression of BCA1 in thymoma cells

Following transfection of Thy0517 cells with FOXN1 siRNA, the expression of FOXN1 mRNA and BCA1 mRNA was 0.230 ± 0.028 and 0.418 ± 0.015, respectively, and was reduced compared with the expression in cells transfected with control siRNA and control cells without siRNA (Table 4). These differences were significant ($P = 0.000$). However, transfection with BCA1 siRNA did not reduce the expression of FOXN1 mRNA (1.222 ± 0.011, $P > 0.05$), but did reduce that of BCA1 mRNA (0.325 ± 0.021, $P = 0.000$) compared with the expression in cells transfected with control siRNA and control cells without siRNA.

Western blot tests showed similar results (Table 5). After transfection of Thy0517 cells with FOXN1 siRNA, the expression of FOXN1 and BCA1 was 0.096 ± 0.001 and 0.117 ± 0.007, respectively, and was significantly reduced compared with the expression in cells transfected with control siRNA and control cells without siRNA ($P = 0.000$). However, BCA1 siRNA could not reduce the expression of FOXN1 (0.583 ± 0.030, $P > 0.05$), but did reduce that of BCA1 (0.112 ± 0.002, $P = 0.000$) compared with the expression in cells transfected with control siRNA and control cells without siRNA.

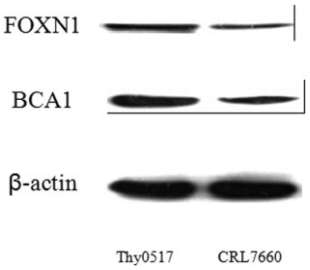


**Figure 4** The protein expression of FOXN1 and B cell-attracting chemokine 1 (BCA1) in Thy0517 and CRL7660 cells, determined using Western blot analysis.

## Expression of FOXN1 and BCA1 may promote proliferation of thymoma cells

To determine the biological significance of FOXN1 and BCA1 in the Thy0517 cell line, MTT cell proliferation assays were performed with and without FOXN1/BCA1 silencing. Similar and significant decreases in proliferation were noted in Thy0517 cells treated with FOXN1 siRNA or BCA1 siRNA for 24, 48, and 72 hours ($P = 0.000$), compared with the control Thy0517 cells. After 72 hours of transfection, the proliferation of thymoma cells had slightly recovered. However, there were no significant differences in cell proliferation between the FOXN1 siRNA and BCA1 siRNA groups at 24, 48, and 72 hours after transfection ($P = 0.202$, 1 and 0.940, respectively). This indicates that FOXN1 and BCA1 have the same effect on Thy0517 cell proliferation (Table 6 and Fig 5).

# Discussion

In humans, FOXN1 is mainly expressed in TECs and keratinocytes, and plays an important role in their develop-

**Table 4** Relative mRNA expression of FOXN1 and BCA1 after transfection

| | FOXN1siRNA | BCA1siRNA | Control siRNA | Control (no siRNA) |
|---|---|---|---|---|
| FOXN1 | 0.230 ± 0.028† | 1.222 ± 0.011 | 1.220 ± 0.004 | 1.249 ± 0.012 |
| BCA1 | 0.418 ± 0.015† | 0.325 ± 0.021† | 1.324 ± 0.009 | 1.652 ± 0.021 |

†Compared with cells transfected with control small interfering ribonucleic acid (siRNA) and control cells without siRNA, $P < 0.05$ (*t*-test). BCA1, B cell-attracting chemokine 1; mRNA, messenger ribonucleic acid.

**Table 5** Relative protein expression of FOXN1 and BCA1 after transfection

| | FOXN1siRNA | BCA1siRNA | Control siRNA | Control (no siRNA) |
|---|---|---|---|---|
| FOXN1 | 0.096 ± 0.001† | 0.583 ± 0.030 | 0.595 ± 0.006 | 0.719 ± 0.001 |
| BCA1 | 0.117 ± 0.007† | 0.112 ± 0.002† | 0.617 ± 0.007 | 0.799 ± 0.001 |

†Compared with cells transfected with control small interfering ribonucleic acid (siRNA) and control cells without siRNA, $P < 0.05$ (*t*-test). BCA1, B cell-attracting chemokine 1.

 

**Table 6** MTT assay to determine the influence of siRNA transfection on Thy0517 cell proliferation

| | A490nm (x ± S) | | | | | | Survival rate (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 24 hours | *P* value* | 48 hours | *P* value* | 72 hours | *P* value* | 24 hours | 48 hours | 72 hours |
| Control cells | 0.31 ± 0.01 | | 0.4 ± 0.01 | | 0.43 ± 0.01 | | 100 | 100 | 100 |
| FOXN1 siRNA | 0.22 ± 0.01 | 0.000 | 0.23 ± 0.01 | 0.000 | 0.31 ± 0.01 | 0.000 | 70.97 | 57.5 | 72.09 |
| BCA1 siRNA | 0.21 ± 0.01 | 0.000 | 0.23 ± 0.02 | 0.000 | 0.31 ± 0.01 | 0.000 | 67.74 | 57.5 | 72.09 |

*Compared with control cells, *t*-test. BCA1, B cell-attracting chemokine 1; siRNA, small interfering ribonucleic acid.

ment and differentiation. During the embryonic stage, the development of primitive TECs into functional cortical TECs (cTECs) and medullary TECs (mTECs) and their interaction requires the presence of sufficient FOXN1 protein. After birth, the FOXN1 protein, which is diversely expressed in TECs, now takes on a different role than at the embryonic stage, and TEC subsets lose FOXN1 with age.[3,14,15] An inborn null mutation in FOXN1 causes a differentiation failure in TECs, thereby halting thymic development at a rudimentary state and completely blocking thymic lymphopoiesis. This causes an alymphoid thymus, severe primary T cell immunodeficiency in nude mice and humans, congenital alopecia, and defective immunity, which all lead to death in early childhood from severe infection.[16] Although the general role of FOXN1 is to regulate the differentiation of epithelial cells in the thymus and skin, it is largely unknown whether the roles of FOXN1 in the thymus and skin are identical. Our immunohistochemical studies showed that the expression of FOXN1 in the thymus is related to the development of thymoma. Approximately 68–76% of thymoma (thymic carcinoma) tissues express FOXN1, which has been defined as a biomarker to differentiate these tumors from other types of tumors in the anterior mediastinum.[4,5] However, the exact role of FOXN1 in the development of thymoma cells is still unknown.

The chemokine BCA1 is a potent lymphoid chemokine that is constitutively expressed in secondary lymphoid tissue.[17] Its expression is associated with secondary lymphoid tissue in rheumatoid arthritis and is associated with the severity of autoimmune disease.[6] High levels of BCA1 have been found in breast, prostate, lung, colon, stomach, oral, and pancreatic tumor tissue, and BCA1 even plays a role in the proliferation and metastasis of tumor cells.[7–10] However, the exact role of BCA1 in the development of thymoma cells is not yet known. The current immunohistochemical studies also showed that the expression of BCA1 was higher in thymoma tissue than that in non-thymomatous thymic tissue.

RNA interference is a process by which RNA molecules, with sequences complementary to a gene's coding sequence, induce degradation of corresponding mRNAs, thus, blocking the translation of the mRNA into protein.[18,19] RNA interference is initiated by exposing cells to long double-strand RNA (dsRNA) via transfection or endogenous expression. Then, dsRNAs are processed into smaller fragments (usually 21–23 nucleotides) of siRNA,[20] which form a complex with the RNA-induced silencing complexes.[21] Introduction of siRNA into mammalian cells leads to downregulation of target genes without triggering interferon responses.[20] Molecular therapy using siRNA has

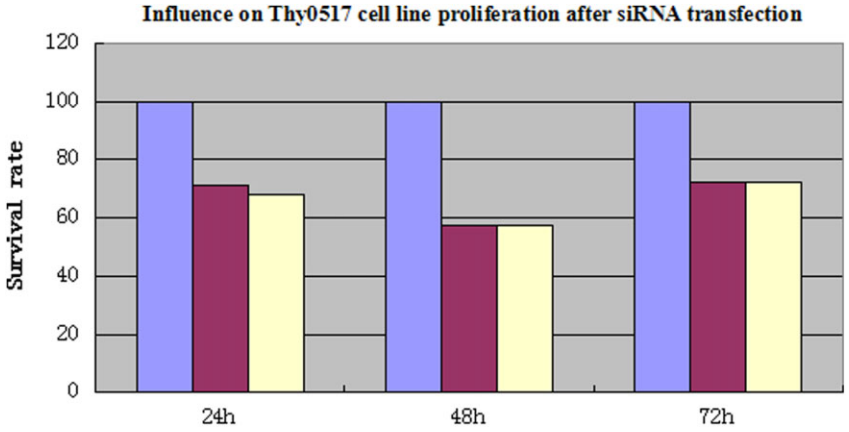


**Figure 5** Survival rate of Thy0517 cells after FOXN1/B cell-attracting chemokine 1 (BCA1) small interfering ribonucleic acid (siRNA) transfection. ▢, control cell; ▢, FOXN1 siRNA; ▢, BCA1 siRNA.

  

shown great potential for diseases caused by abnormal gene over-expression or mutation, such as various cancers, viral infections, and genetic disorders.

In this study, the expression levels of FOXN1 and BCA1 were found to be higher in thymoma tissue than in non-thymomatous tissues. However, the roles of these two factors during thymoma tumorigenesis need to be clarified. For this purpose, the thymoma cell line Thy0517 and normal TEC cell line CRL7660 were used to compare the expression of FOXN1 and BCA1. The RT-PCR and Western blot results showed that the expression of both FOXN1 and BCA1 was higher in Thy0517 cells than in CRL7660 cells. Therefore, Thy0517 was chosen as the optimal cell line for this siRNA research.

Following transfection of Thy0517 cells with FOXN1 siRNA and BCA1 siRNA, RT-PCR and Western blot showed that the inhibition of FOXN1 expression downregulated the expression of BCA1, while the inhibition of BCA1 did not induce the same effect on FOXN1. This finding demonstrates that FOXN1 acts as an upstream gene and positively regulates the expression of BCA1.

In initial immunohistochemical studies, we found that the expression of FOXN1 and BCA1 in thymoma cells was higher than in normal thymic tissue, which supports the results for the thymoma cell line and normal TECs in this research. These findings indicate that FOXN1 and BCA1 may play a role in the development of thymomas. In this study, MTT assays showed that in the Thy0517 cell line, the inhibition of FOXN1 and BCA1 suppressed the proliferation of thymoma cells. Following transfection with FOXN1 siRNA/BCA1 siRNA, the survival rate of thymoma cells was significantly reduced compared with that of control group cells in the first 24, 48, and 72 hours. However, there were no significant differences between the FOXN1 siRNA and BCA1 siRNA group cells. The influence on cell proliferation was limited, as thymoma cell proliferation began to recover at 72 hours after transfection with FOXN1/BCA1 siRNA. This demonstrates that FOXN1 and BCA1 may partly promote the proliferation of thymoma cells via the same signaling pathway, and that FOXN1 acts upstream of BCA1.

## Conclusion

FOXN1 and BCA1 expression was higher in thymoma tissue samples and cell lines than in non-thymomatous tissue and normal thymic epithelial cells. FOXN1 acts upstream of BCA1 and both FOXN1 and BCA1 promote thymoma cell proliferation. Further work on microarray and animal experiments is needed to clarify the abnormally expressed genes related to the development and proliferation of thymoma cells and the role played by FOXN1 and BCA1.

## Acknowledgment

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## Disclosure

No authors declare any conflict of interest.

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