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Research article

RNA INTERFERENCE AGAINST Biot2, A NOVEL MOUSE TESTIS - SPECIFIC GENE, INHIBITS THE GROWTH OF TUMOR CELLS

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Abstract: Biot2 is a novel murine testis-specific gene that was first identified using the SEREX technique, and named by our laboratory. Using conventional RT-PCR and real time RT-PCR, we tested the expression profile of Biot2 in normal tissues and various murine tumor cell lines. Using RNA interference, we studied the biological function of Biot2 in tumorigenesis. We applied various types of growth assay, such as the *in vitro* MTT, colony-forming and BrdU incorporation assays, along with *in vivo* tumorigenicity assays, to reveal its inhibition of tumor cell proliferation. The results revealed that the Biot2 transcript was detected only and strongly in the testis tissues and abundantly in

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Abbreviations used: ATCC – American Type Culture Collection; BrdU – bromodeoxyuridine; B16 – murine melanoma cell; CT antigen – cancer/testis antigen; CT26 – murine colorectal adenocarcinoma cell; Hepa and H22 – murine hepatocellular carcinoma cell; LL/2 – murine Lewis lung carcinoma cell; MethA – murine fibrosarcoma cell, NCBI – National Center for Biotechnology Information; OD – optical density; ORF – open reading frame; SD – standard diviation; SF – survival fraction; shRNA – short hairpin RNA; 4T1 – murine breast cancer cells

five types of murine cancer cell line. Treating B16 murine melanoma, LL/2 murine Lewis lung carcinoma and CT26 murine colorectal adenocarcinoma with special shRNA targeting Biot2 can significantly reduce the proliferation rate of these three tumor cell lines *in vitro*, as measured by the MTT, colony-forming and BrdU incorporation assays. The tumorigenicity of the CT26 cells transfected with special shRNA targeting Biot2 was also decreased distinctly *in vivo* compared with the control. It was therefore concluded that Biot2 plays a key role in tumorigenesis and could be a potential target for biotherapy.

Key words: RNA interference, Biot2, Testis-specific, Proliferation

INTRODUCTION

Male germ cells share some characteristics with tumor cells, as they are both types of primary differentiation cells [1]. Some cancer antigens have been found to be testis-specific. This group of antigens is referred to as the cancer/testis antigen family (CT antigens), with the members sharing the following features: (i) mRNA expression predominantly in the testis, and generally absent in other healthy tissues; (ii) gene activation and high-level mRNA expression in certain malignant tumors; and (iii) expression in malignancies in a lineage-nonspecific fashion [2-5]. It is believed that these antigens may escape normal immune recognition. The identification of CT antigens is a growing area of research, as they may serve as tumor-restricted antigens for the development of vaccines with a lower risk of pre-existing immune tolerance and of potential autoimmune reactions in healthy tissues. Various methods have been applied to isolate cancer testis antigens. Of these, the serological analysis of recombinant cDNA expression libraries (SEREX) technique has prominent advantages. For example, it is not restricted to antigens expressed on the cell surface, but is also able to detect the immune response to intracellular antigens. The development of the SEREX technique will open new avenues for the identification of CT antigens, including the MAGE family (MAGE-1, MAGE-3), GAGE-1, BAGE, HOM-MEL-40 (*SSX2*), and NY-ESO-1 [6-10].

In this study, we attempted to isolate novel heterogenous cancer testis antigens, using the SEREX method on a mouse testis cDNA library and serum from rabbits immunized with human ovary cancer cells. One of the isolated antigens, Biot2, was found to be expressed only in the testis among the normal tissues, but is more widespread in various tumor cell lines. To better understand its function, we cloned its gene from the testes of 6-week old C57 mice, synthesized three short hairpin RNAs (shRNAs) targeting the Biot2 plasmid vectors, and then introduced them into CT26 cells. The 3#-shRNA was efficiently and specifically able to knock down Biot2 expression in transiently transfected CT26 cells. Furthermore, we detected that the proliferation of CT26 cells, LL/2 cells and B16 cells treated with 3#-shRNA *in vitro* was significantly inhibited, and that the tumorigenicity of CT26 cells transfected with 3#-shRNA *in vivo* was

evidently reduced. This is the first paper to describe the function of Biot2 on oncogenesis, and these results will facilitate further investigation.

MATERIALS AND METHODS

Bioinformatics analysis

Biot2 was localized to chromosome 8 by aligning the cDNA sequence to the NCBI (National Center for Biotechnology Information) and Ensemble databases. The exon-intron structure was deduced by comparing the genomic and cDNA sequences using Blat Search Genome Software. We translated the program in ExPASy to identify the ORF (Open Reading Frame). Amino acid homologies between different species were analyzed using the GENESTAR program, and the percentage homologies were calculated. We compared the homology in the entire coding region and the CCDC7 domains. Generic phosphorylation sites and kinase-specific phosphorylation sites were predicted with the NetPhos 2.0 and NetPhosK 1.0 servers. TMHMM was used to predict transmembrane helices in the peptide. PROSITE and InterProScan were used to predict various protein patterns and profiles. PSORT II was used to predict protein sorting signals and intracellular localization.

Animal and cell lines

Male 6-week old C57BL/6 mice and female 6- to 8-week old BALB/c mice were obtained from the Laboratorial Animal Center of Sichuan University. The animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Sichuan University and the National Research Council Guide for the Care and Use of Laboratory Animals. B16 murine melanoma, Hepa and H22 murine hepatocellular carcinoma, MethA murine fibrosarcoma, 4T1 murine breast cancer and LL/2 murine Lewis lung carcinoma cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and grown in DMEM supplemented with 10% FBS. CT26 murine colorectal adenocarcinoma cells were purchased from ATCC and grown in RPMI medium 1640 with 10% FBS. These tumor cell cultures were maintained in a 37ºC incubator with a humidified 5% CO_2 atmosphere.

Isolation of total RNA

All the male C57BJ/6 mice were killed by neck dislocation, and then selected tissues (brain, heart, muscle, spleen, liver, kidney, bladder, testis, lung, intestine, bone marrow, thymus, macrophage and blood) were dissected, frozen in liquid nitrogen, and stored at -80ºC. Six types of murine tumor cells were collected after digestion with pamcreatin and washing with PBS, and stored at -80ºC. The total RNA from these tissues and the tumor cells was isolated using the TRIzol reagent (Invitrogen).

Semi-quantitative reverse transcription (RT-PCR)

Reverse transcription was done with the SuperScript II One-Step RT-PCR System (Takara) following the manufacturer's protocols. The following sequences were used: the Biot2 forward primer: 5'-GGATCCAAAATGAAA TGTGCAAAGCATCC-3' and reverse primer: 5'-CTCAGCACCAATGAAG CCCTCTCTT-3'; and the GAPDH (used as a control) forward primer: 5'-CCC TTCATTGACCTCAACTA-3' and reverse primer: 5'-CCAAAGTTGTCAT GGATGAC-3'. The following RT-PCR conditions were used: an initial denaturation step at 50ºC for 50 min, 94ºC for 4 min, followed by 35 cycles at 94ºC for 45 sec, 58ºC for 45 sec, and 72ºC for 45 sec, and a final elongation step at 72ºC for 10 min. The RT-PCR fragment was then cloned into pMD18-T vectors (TaKaRa, Japan) and sequenced (Corporation Invitrogen, China).

Real time RT-PCR

The total RNA from 13 types of tissue from adult C57 mice and 6 types of mouse tumor cell, prepared as described above, were analyzed for the expression of Biot2 mRNA using the ExScript RT-PCR Kit (Perfect Real Time) (TaKaRa), according to the protocol. First, 500 ng total RNA was reverse transcribed using 50 U ExScript RTase and 25 pmol Oligo dT for 15 min at 42ºC and 2 min at 95ºC. Then, the quantitative real time PCR analysis of the Biot2 transcript was performed using the ABI PRISM 7000 apparatus (Applied Biosystems) with the following primers: 5'-CCATTTGCAGTGGCAAAG-3' and 5'-CACCCCATTT GATGTTAGTG-3'. 2 ul cDNA of the above reaction system was amplified with 2 ul SYBR Green I dilution and 0.4 ul ROX Reference Dye solution for 10 sec at 95ºC, then for 45 cycles for 5 sec at 95ºC, and 30 sec at 55ºC. Accurate amplification of the target template was checked by performing a melting curve. Parallel amplification of the GAPDH transcript was carried out to normalize the expression data of the Biot2 transcript. The results were calculated using iCycler IQ Real Time Detection System software (Bio-Rad).

Synthesis of shRNA against Biot2

Three shRNAs against Biot2 and the negative control HK were synthesized by the Wuhan Genesil Biotechnology Corporation (China). The respective sequences of Biot2 1#-shRNA, 2#-shRNA and 3#-shRNA were 5'-CCGAAUG ACUUAAGUAGUG-3', 5'-ACGGUUCAGUUUCAUACCA-3' and 5'-AACA UGCGAAACGGAACGG-3'. The oligonucleotide pairs were designed to contain a terminal *BamH*I or *Hind*III restriction site for subcloning into the *BamH*I or *Hind*III site of the Gensile-1 vector to generate Gensile-1 Biot2-shRNA vectors. These vectors produced an shRNA with a TTCAAGACG linker sequence that formed looped structures. This linker was processed with Dicer to generate a Biot2-specific siRNA. A negatively scrambled siRNA control, HK, with no significant homology to the mouse gene sequences, was designed to detect non-specific effects. The sequence of HK is 5'-CUGAAGUAUUCCGCGUACG-3'.

Screening shRNA against Biot2

1#-shRNA, 2#-shRNA, 3#-shRNA and the negative control HK were transfected into CT26 cells with lipofectamine 2000. Semi-quantitative RT-PCR and real time RT-PCR were carried out with total RNA from these cells to detect the expression of Biot2 at the mRNA level, because of the absent polyclonal antibody of Biot2. The shRNA which produced the strongest inhibition of expression of Biot2 was considered the most effective one, and was used in the further experiments. The negative control shRNA HK was used for all the procedures simultaneously.

MTT assay

Three groups were selected for the study: untreated control cells; cells transfected with the negative control HK; and cells transfected with 3#-shRNA. Murine tumor cells (CT26, B16, LL/2) were prepared in medium at a concentration of 3×10^4/ml, and 0.1 ml of cell suspension was placed in each well of a 96-well plate. After transfection for 48 h, an MTT solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide was added to the cells at a final concentration of 500 μg/ml and incubated continuously at 37ºC for 4 h. After removing the solution, DMSO was added to dissolve the crystals. RPMI 1640 or DMEM medium served as a blank control group, with the untreated cells as a control group. The value of the optical density (OD) was obtained from the differences in absorbance at a wavelength of 490 nm, and the suppression rate was calculated with the following formula: Suppression rate = [(OD value in the experimental group - OD value in the blank control group)/(OD value in the control group - OD value in the blank control group)] × 100%.

Cell colony-forming assay

After 24 h, cells transfected with 3#-shRNA or HK were seeded in 6-well plates with 200 cells per well, and cultured in routine medium for 10 to 14 days. All the plates were incubated at 37ºC in a humidified atmosphere of 5% CO_2. The colonies were fixed with 70% ethanol, stained with crystal violet, and counted. Colonies containing more than 50 cells were considered viable. The results were expressed as the means of the SF (survival fraction) ±SD (standard diviation) of triplicate samples. The SF was calculated with the following formula: SF(%) = (colony number for the experimental group/colony number for the control group) × 100%.

BrdU incorporation

DNA synthesis was measured using a bromodeoxyuridine (BrdU) ELISA kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. Briefly, sub-confluent cells were treated with the negative control HK or 3#-shRNA for 48 h. The cells were labeled with BrdU and incubated with anti-BrdU antibody. BrdU incorporation was detected using a colorimetric substrate solution, and the absorbance was measured at 370 nm. The results are presented as the mitotic index, defined as the percentage of BrdU-positive nuclei per number of cells.

Tumorigenicity assay

Twenty 6- to 8-week old female BALB/c mice were used. The mice were divided into four groups with five mice per group. The experimental group was inoculated with CT26 cells transfected with 3#-shRNA. The three control groups were injected with CT26 cells transfected with HK, liposome, or PBS. The resulting tumors were measured twice every week, and the tumor volumes (mm) were calculated using the standard formula: length × width^2 × 0.5326. The animals were killed 28 days after inoculation, and the tumors were excised and weighed. The statistical analysis was performed using the Student's t test. The tissues were frozen in liquid nitrogen, and stored at -80ºC or preserved in neutral buffered formalin.

RESULTS

Identification of the mouse Biot2 cDNA and peptide sequences

The cDNA of Biot2 was obtained through RT-PCR from the testes tissue of C57 mice, yielding a single band of the predicted size (0.596 kb). Using the translating program in ExPASy, we identified the ORF of Biot2. Thus, we concluded that we had obtained the full cDNA sequence. This sequence was submitted to the GenBank and MGI databases. The GenBank accession number of the sequence is EF100607. By aligning the cDNA sequence to the NCBI and Ensemble databases, it was deduced that the Biot2 gene is localized on chromosome 8 and contains 6 exons and 5 introns. Furthermore, the nucleotide sequence of the boundaries between the exons and introns follows the GT-AG rule. The CDS region begins at the third exon. The Biot2 gene encodes a 160 amino acid residues polypeptide. An alignment analysis of different species including rat, human, monkey and cattle indicated that the amino acid sequences are highly conservative (alignment > 50%; Fig. 1). Furthermore, these proteins all have coiled coil domains or similar, suggesting that they would be involved in protein-protein interaction.

The expression pattern of Biot2

The expression of Biot2 in various tissues was studied by conventional RT-PCR and real time RT-PCR. The results (Fig. 2) showed that Biot2 was exclusively expressed in the testes but not detected in the other 13 tissues sampled from adult C57 mice. Biot2 was also highly transcribed in 5 cancer cell lines from the epithelium (B16 murine melanoma, Hepa and H22 murine hepatocellular carcinoma, CT26 murine colorectal adenocarcinoma, 4T1 murine breast cancer and LL/2 murine Lewis lung carcinoma), but absent in MethA murine fibrosarcoma. GAPDH was used as the control. Each real time RT-PCR experiment was done in triplicate, and the mean CT value was used for data analysis. The final result was presented as the mean of three separate experiments ± SD.

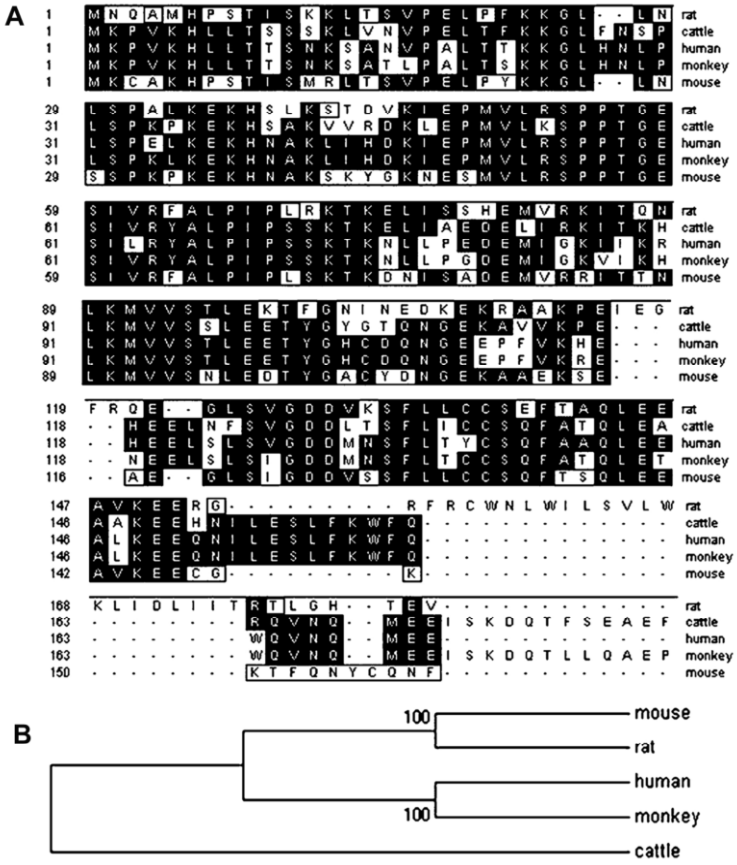

Fig. 1. A sequence comparison of the Biot2 protein with other CCDC7 class proteins. A – A comparison of mouse Biot2 protein with the rat, monkey, cattle and human homologues. The dark shadows indicate identical amino acids and the blanks indicate different amino acids. Dots represent the absent amino acids. B – A schematic illustration of the evolutionary relationships. The putative phylogenetic tree was constructed using the GENESTAR program.

Screening of the specific shRNA-Biot2

Semi-quantitative conventional RT-PCR and real time RT-PCR analysis revealed that the expression of Biot2 was inhibited more distinctly by the 3#-shRNA than by the other two shRNAs (Fig. 3). The inhibition ratios of the 1#-, 2#- and 3#-shRNA were respectively up to about 66.7%, 40.5% and 90%. 3#-shRNA was selected as the most efficient shRNA targeting Biot2, and was used in further investigations.

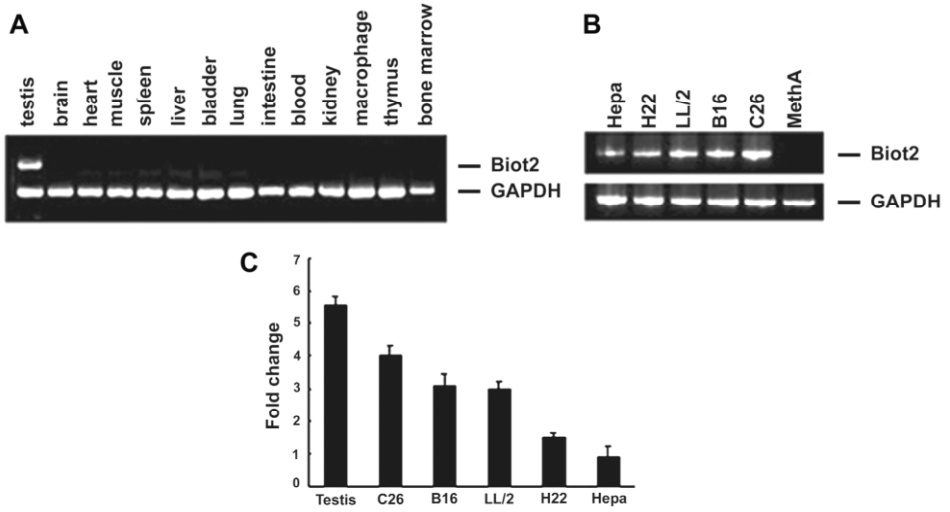

Fig. 2. The expression pattern of Biot2 in multiple tissues and varied tumor cells. A, B – RT-PCR was performed with Biot2-specific primers and 0.5-1 mg total RNA from 14 different tissues of the adult C57 mouse: brain, heart, muscle, spleen, liver, kidney, bladder, testis, lung, intestine, bone marrow, thymus, macrophage, blood (A); and 6 different mouse tumor cells: Hepa, H22, LL/2, B16, CT26 and MethA (B). GAPDH transcripts were used as a control. C – The quantitative analyses of the Biot2 transcript in the adult testis and various tumor cells detected by real time RT-PCR. The expression level of the Biot2 transcript was normalized to that of the GAPDH transcript, which was measured in the same cDNAs. The values were the ratios of Biot2 transcript to GAPDH transcript. The data was obtained from triplicate experiments and is indicated as means ± SD.

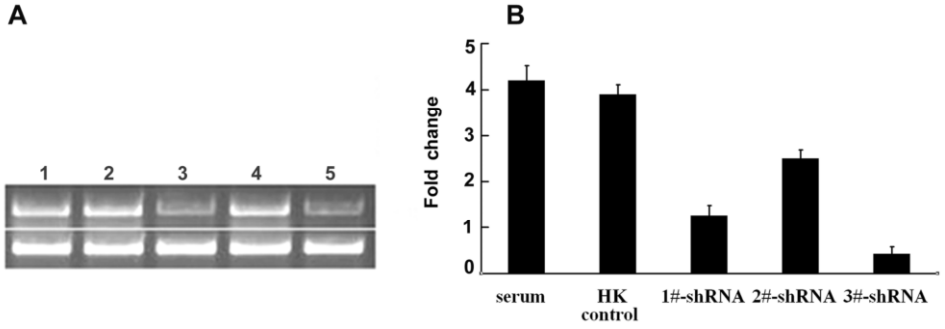

Fig. 3. Screening of the specific shRNA-Biot2. Conventional RT-PCR (A) and quantitative real time RT-PCR (B) were used to detect the expression of Biot2 in CT26 cells treated with HK and with 1#-, 2#- and 3#-shRNA. The wild-type CT26 cells acted as a control. It showed that 3#-shRNA was the most efficient shRNA targeting Biot2. In Fig. 3A, lanes 1 through 5 were respectively represented by wild-type CT26 cells, CT26 cells transfected with HK, and CT26 cells transfected with 1#-, 2#- and 3#-RNA.

Inhibition of the tumor growth *in vitro*

Tumor cells (CT26, B16 and LL/2) cultured in a 96-well plate were transfected with HK or 3#-shRNA for 48 to 72 h. The rate of cell proliferation was then measured using the MTT assay (absorbance), which was significantly lower in tumor cells treated with 3#-shRNA than in untreated and HK-treated tumor cells (Fig. 4A). Three cell lines showed a strong decrease in absorbance after treatment with 3#-shRNA. The inhibition of proliferation was around 50%, which was consistent with the morphological change of CT26 transfected with 3#-shRNA observed under the microscope (Fig 4B): a rounded, granulated morphology, and a tendency for a large number of the cells to detach from the

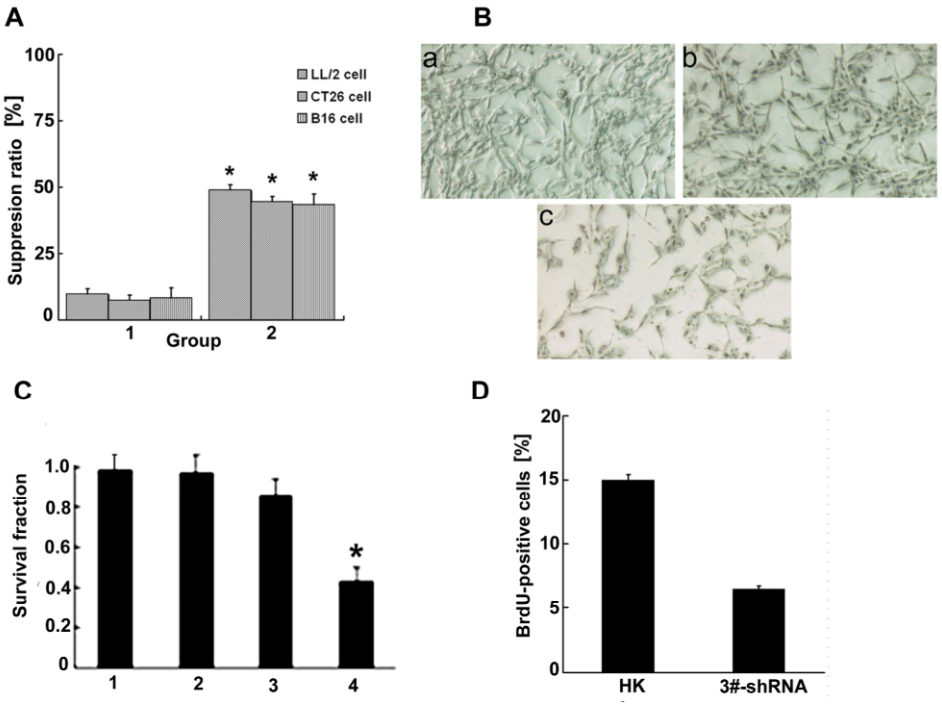

Fig. 4. Experiments on the inhibition of tumor growth of 3#-shRNA targeting Biot2 *in vitro*. A – The proliferation activity of the tumor cells was detected using the MTT assay. The data for the wild-type cells is presented as a control (inhibitor ratio = 0). B – The morphology change of CT26 transfected with 3#-shRNA after 48 h. a – wild-type CT26 cells; b – CT26 cells transfected with HK; c – CT26 cells transfected with 3#-shRNA. C – Cell viability was detected with the colony-forming assay. D – DNA synthesis was examined with the BrdU incorporation assay.

culture flasks. Meanwhile, to confirm the effect of 3#-shRNA on growth inhibition, the BrdU incorporation and colony-forming assays were conducted at 48 h post-transfection. The colony-forming assay was universally recognized as the standard for measuring the effects on cell viability. In our studies, the

survival fraction was about 40% in the 3#-shRNA-transfected cells, compared with the control. The difference was statistically significant ($P < 0.05$; Fig. 4C). The BrdU incorporation assay was performed to examine the effect of 3#-shRNA on DNA synthesis in CT26 cells. It was found that 3#-shRNA inhibited DNA synthesis by ~60%, compared with the control HK (Fig. 4D).

Inhibition of the tumor growth *in vivo*

CT26 cells were transfected with either 3#-shRNA or the negative control HK. 16 h post-transfection, the wild-type CT26 cells, and the CT26 cells transfected with 3#-shRNA or HK were inoculated into BALB/C mice. It was found that the tumorigenicity of the experimental group decreased in an obvious manner, and the weight and volume of the tumors were drastically reduced compared with the measurements for the controls (Fig. 5). The difference was statistically significant ($P < 0.05$).

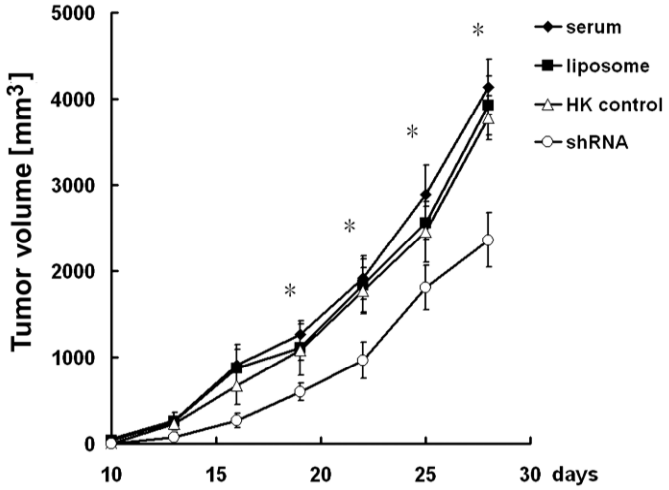

Fig. 5. Analysis of the effect of 3#-shRNA on tumorigenecity *in vivo* ($p < 0.05$). ◆ – wild-type CT26 cells; ■ – CT26 cells transfected with lipofectamine 2000; Δ – CT26 cells transfected with HK; ○ – CT26 cells transfected with 3#-shRNA.

Our findings indicated that the Biot2 gene's special shRNA significantly inhibited the growth of CT26 cells, LL/2 cells and B16 cells *in vitro* and inhibited the growth of CT26 cells *in vivo*.

DISCUSSION

Cancer/testis antigens display a predominant expression in various types of cancer cells and undetectable expression in normal tissues other than the germ cells of the testis. Their tumor-restricted expression suggests that they are usable

as the target antigens for cancer immunotherapy without autoimmune reactions that could potentially harm normal tissues. For example, some cancer/testis antigens, like NY-ESO-1, are applied as cancer vaccines in early clinical trials for breast cancer patients and malignant melanoma patients [11-15], and NY-BR-1, as a mammary gland differentiation antigen, is a target for cancer immunotherapy, and will be applied as a potential target for antibody-based therapies in breast cancer [16-20].

Originally described in 1995, the serological analysis of recombinant cDNA expression libraries (SEREX) has become a powerful approach to identify immunogenic tumor antigens. In this report, we applied the SEREX method with a mouse testis cDNA library and serum from rabbits immunized with human ovary cancer cells in order to obtain novel heterogenous cancer antigens which could provide new strategies for cancer immunotherapy. One of the isolated novel antigens was Biot2, which encoded a putative protein of *M*r 17,736, having no homology with any known protein. With the bioinformatics analysis, it was found that Biot2 was highly conservative in various mammals such as rat, human, monkey and cattle (alignment > 50%). These proteins all had coil-coil domains or similar, which indicated that they would have the similar biological function involving protein-protein interaction. The interspecies conservation suggested that Biot2 might be an essential gene for evolution. Furthermore, our previous studies and the bioinformatics analysis revealed that rat Biot2 might correlate with sperm development and with the potential function to stimulate the proliferation of cells [21]. There were also expression differences of human Biot2 between cancerous and adjacent normal tissues in the endometrial cancer group and the normal endometrial group, which revealed the potential function of the human homologous gene on carcinogenesis [22]. To investigate whether it played a role in proliferation and carcinogenesis, we first tested the expression of mouse Biot2 in normal tissues, because studying the expression profile of a novel gene could give a hint of its function. Conventional RT-PCR and real time RT-PCR analysis revealed that as far as normal tissues were concerned, the Biot2 transcript was detected only and strongly in the mouse testis, but abundantly in all five types of mouse cancer cell line derived from epithelial cells. It was absent in the sarcoma cell line. The differential expression profile of the Biot2 gene correlated with the characteristics of the CT antigen, which suggested that Biot2 would become a goal in cancer immunotherapy. Furthermore, it was indicated that Biot2 would play an essential role in the process of tumorigenesis and be involved in the regulation of tumor cell growth. Therefore, we synthesized three shRNAs targeting Biot2 and introduced them into CT26 cells. The 3#-shRNA was the most efficiently and specifically able to knock down the expression of the Biot2 gene in transiently transfected CT26 cells, as detected by semi-quantified RT-PCR and real time RT-PCR analysis. The MTT assay showed that down-regulation of Biot2 at the level of transcription obviously suppressed the proliferation of B16 cells, CT26 cells and LL/2 cells, which had high-level mRNA expression of Biot2 under normal

conditions, compared with the control. The effect of 3#-shRNA on cell growth was also confirmed using the colony-forming and BrdU incorporation assays. It was detected that the cell viability and DNA synthesis were all inhibited by 40-50% in CT26 cells treated with 3#-shRNA, compared with the control. The difference was statistically significant ($P < 0.05$). Furthermore, *in vivo*, the tumorigenicity of CT26 cells transfected with 3#- shRNA was decreased obviously, and the weight and volume of tumors were drastically reduced compared with those of tumors formed by the other three control groups. To further study the response of CT26 cells to 3#-shRNA, apoptosis was measured by annexin V staining *in vitro*. However, there was no significant difference between the cells of the control and experimental groups. Therefore, it was supposed that the effect on cell growth of 3#-shRNA targeting Biot2 would be mostly attributed to the inhibition of proliferation and would be unconcerned in the direct promotion of apoptosis death.

RNA interference (RNAi) has been shown to have great potential in the fields of gene function and gene therapy [23-24]. Our findings indicated that shRNA specifically targeting the Biot2 gene significantly inhibits the growth of tumor cells both *in vitro* and *in vivo*, which showed promise as a precise means for the disruption of gene expression to achieve a therapeutic effect. For a further understanding of the functions of Biot2 and its role in the tumorigenesis, the cDNA microarray analysis, the interaction proteins or even knockout mice would also be of interest.

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