In vitro study on shRNA-mediated reduction of testis developmental related gene 1 expression and its effects on the proliferation, invasion and apoptosis of NTERA-2 cells

YU GAN, JIANFU YANG, YONG WANG, ZHENGYU TAN, XIANZHEN JIANG and YUXIN TANG

Department of Urology, The Third Xiangya Hospital of Central South University, Changsha, Hunan 410013, P.R. China

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Abstract. Testis developmental related gene 1 (*TDRG1*) is a novel human testis-specific gene. *TDRG1* is differentially expressed in cancerous tissue compared with normal testicular tissue and demonstrates a unique expression pattern in normal testes; therefore, this gene may be involved in the occurrence and development of testicular germ cell tumors (TGCT). In the present study, the expression level of *TDRG1* was downregulated in human TGCT NTERA-2 cells by RNA interference (RNAi) in order to investigate the association between TDRG1 and TGCT. The TDRG1 mRNA and protein expression levels in NTERA-2 cells were significantly inhibited following transfection with specific RNAi plasmids. The ability to proliferate (inhibited by 15.4% at day 3 and 26.1% at day 5; P<0.001) and invade (reduced by 49.1%; P=0.01) *in vitro* was suppressed in cells in which the expression level of *TDRG1* was reduced, and a corresponding increase in the apoptotic potential was observed (the early apoptotic potential and total apoptotic potential were increased by 75%; P=0.019 and 54.8%; P=0.009, respectively). The results of the present study indicated that the biological behavior of NTERA-2 cells is associated with *TDRG1* expression levels, and that this gene may be a novel target candidate in the treatment of TGCT.

Introduction

Testicular germ cell tumors (TGCTs) are a type of solid tumor that are most commonly observed in young men aged 15-35 years (1). There is a relatively large difference in the incidence of TGCT among different regions or different races, but the worldwide incidence of TGCT has increased in the last 30-40 years (2,3). However, >95% patients diagnosed at an early stage are cured (4). TGCT treatment is associated with chronic side effects; patients with advanced-stage cancer are less sensitive to comprehensive treatments, resulting in a poor prognosis (5). The molecular biology of TGCT remains largely unknown, but the onset and development of the disease is a process that involves multiple genes (6). Increased understanding of the molecular biology of TGCT is essential in order to define targeted treatments and identify novel markers for diagnosis and prognosis.

Testis developmental related gene 1 (*TDRG1*; GenBank ID, DQ168992) is specifically expressed in human testicular spermatogenic cells, but not in non-reproductive tissues. Furthermore, expression levels of *TDRG1* are relatively increased in young men aged 15-33 years (7). Notably, all TGCT originate from the spermatogenic cells and the high-risk ages for the development of TGCT are consistent with the expression pattern of *TDRG1*. Previous studies have demonstrated the abnormal expression of TDRG1 protein in TGCT compared with non-malignant human testicular tissues (8). Therefore, it is possible that TDRG1 may be associated with the onset and development of TGCT.

The use of short hairpin (sh)RNA to silence a target gene has become a conventional and accurate method (9). In the present study, RNA interference (RNAi) was used to downregulate the expression level of *TDRG1* in TGCT NTERA-2 cells. The effects of reduced *TDRG1* expression levels on NTERA-2 cells were observed. The biological behavior of NTERA-2 cells with reduced *TDRG1* expression was detected and compared with control groups and the association between these biological behaviors and *TDRG1* was analyzed.

Materials and methods

Cell culture. The human TGCT NTERA-2 cell line was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA, USA) at 37˚C in a humidified atmosphere of 5% CO_2.

TDRG1-targeting siRNA expression vector. Two previously constructed recombinant plasmid vectors were used in the present study (10). The recombinant plasmids were named

Correspondence to: Professor Yuxin Tang, Department of Urology, The Third Xiangya Hospital of Central South University, 138 Tongzipo Road, Changsha, Hunan 410013, P.R. China
E-mail: rainson27@126.com

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pGPU6/GFP/Neo-shRNA486 (psh486) and pGPU6/GFP/Neo-shRNA-control (pshneg), respectively. The DNA concentration and purity of the plasmids was detected by ultraviolet spectrophotometry (SmartSpec 3000; Bio-Rad Laboratories, Inc., Hercules, CA, USA) then they were stored at -20˚C. The shRNA486 sequence (the mRNA coding region of 486-506 is in upper case): Sense 5'-caccGCGCAGGATCAAGC TACAATGttcaagagaCATTGTAGCTTGATCCTGCGCtttttt g-3' and antisense 5'-gatccaaaaaaGCGCAGGATCAAGCTA CAATGtctcttgaaCATTGTAGCTTGATCCTGCGC-3'. The negative control sequence (the target sequence that has no homology with the human gene is in upper case): Sense, 5'-caccGTTCTCCGAACGTGTCACGTCcaagagaTTACGTGA CACGTTCGGAGAAttttttg-3' and antisense 5'-gatccaaaa aaTTCTCCGAACGTGTCACGTAAtctctTGACGTGACAC GTTCGGAGAAc-3'.

Transfection and grouping. NTERA-2 cells were passaged with 0.25 g/l trypsin (GE Healthcare Life Sciences, Logan, UT, USA) and seeded into 6-well plates at a density of $4.5x10^4$ cells/well. When the cells reached ≥70% confluence, they were transfected in 3 groups as follows: i) Control group (no transfection); ii) negative control group (pshneg transfection); and iii) shRNA486 group (psh486 transfection).

Following the Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) manufacturer's instructions, the plasmids described above and the liposome were diluted separately with Opti-MEM (Invitrogen Life Technologies). The effective ratio of plasmid:liposome was 1:2.5 (μg:μl). The diluted plasmid and liposome were mixed and incubated at room temperature for 20 min to allow complex formation. The transfection mixture was then added to each well with 2 ml of FBS-free DMEM. Following 6 h incubation at 37˚C, the mixture was replaced with complete medium. Fluorescence microscopy (Nikon E800; Nikon Corporation, Tokyo, Japan) was used to confirm successful transfection after 48 h.

RNA extraction and fluorescence quantitative polymerase chain reaction (qPCR). The specific primers designed to amplify *TDRG1* and *GAPDH* were designed and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The following primer sequences were used: TDRG1, sense 5'-GAAGAGGAG GGAGGCAGTCT-3' and antisense 5'-GCCCAATTCCTC TTGACTGA-3'; GAPDH, sense 5'-ACCACAGTCCATGCC ATCAC-3' and antisense 5'- TCCACCACCCTGTTGCTG TA-3'. Total RNA was extracted from NTERA-2 cells using TRIzol reagent (Invitrogen Life Technologies). First strand cDNA synthesis was performed with the RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Fluorescence qPCR was performed in a total volume of 25 μl containing 1 μl cDNA, 100 nM of each TDRG1 or GAPDH primer, 12 μl 2X SYBR Green PCR Master mix (Thermo Fisher Scientific, Inc.) and ddH_2O to a total volume of 25 μl. All the procedures were performed according the manufacturer's instructions. The reaction parameters were 95˚C for 5 min (pre-denaturation), followed by 20 sec each at 94˚C (denaturation), 56˚C (annealing) and 72˚C (elongation) for 40 cycles, then a final elongation step for 5 min at 72˚C. Preliminary experiments verified that the amplification efficiency of the target gene was similar to that of the reference gene GAPDH. The $2^{-\Delta\Delta Ct}$ method was used for relative quantification and statistical analysis (11). Each sample was set up as three duplications and tested in triplicate.

Immunofluorescence. The cells cultured on glass coverslips were transfected according to the groups outlined previously. At 1, 3 or 5 days post-transfection, the cells were washed twice with phosphate-buffered saline (PBS) and fixed with ice cold 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 20 min at room temperature, air-dried, and washed with PBS 3 times. The cells were then exposed to 0.25% Triton X-100 (Sigma-Aldrich) for 20 min. Normal goat serum blocking fluid (10%; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was applied to the fixed cells for 20 min at room temperature and then the blocking fluid was removed. Mouse anti-human TDRG1 monoclonal antibody (12) (1:1,000; ProMab Biotechnologies, Inc., Richmond, CA, USA) was applied to the cells and incubated overnight at 4˚C (12). The cells were then washed with PBS 3 times, the secondary antibody Cy3-conjugated goat anti-mouse IgG (1:200; cat. no. A0521; Beyotime Institute of Biotechnology, Shanghai, China) was applied to the cells and the cells were incubated in the dark at 37˚C for 40 min. The cells were then washed with PBS 3 times, and stained with DAPI (100 ng/ml; Beijing Solarbio Science & Technology Co., Ltd.), and the cells were again rinsed with PBS 3 times. The cells were observed using a fluorescence microscope (Nikon E800) and the integrated optical density (IOD) of the TDRG1 protein expression in 10 randomly selected visual fields was measured using 'Motic Fluo' software, version 1.0 (Motic China Group Corporation, Ltd., Shenzhen, China).

Cell proliferation assay. The effect of TDRG1 on the proliferation of NTERA-2 cells was measured using MTT (Sigma-Aldrich) assay. The transfected cells were cultured in 96-well plates at a density of ~$1x10^4$ cells/well in a volume of 100 μl. At time points of 1, 3 or 5 days, 20 μl 5 mg/ml MTT solution was added to each well and the cells were incubated for 4 h at 37˚C. The supernatant in each well was aspirated and discarded and 150 μl dimethyl sulfoxide (Amresco, LLC, Solon, OH, USA) was added to dissolve the formazan. A microplate reader (WD-2102A; Beijing Liuyi Instrument Factory, Beijing, China) was used to detect the OD value for each well at a wavelength of 570 nm. Each sample was tested in triplicate.

Cell invasion assay. The *in vitro* invasion capability of the cells was measured using the Transwell invasion assay. The transfected cells were seeded at a density of ~$1x10^5$ cells/well in the top chamber with a membrane (6-well insert, 8-μm pore size; Corning Incorporated, New York, NY, USA) coated in Matrigel (1 mg/ml; BD Biosciences, Franklin Lakes, NJ, USA). The cells were cultured in serum-free medium, and medium containing 10% FBS was used as a chemoattractant in the lower chamber. Following 24-h incubation, the chamber was removed, the Matrigel and the cells in the upper chamber were wiped with a cotton swab and discarded. The cells in the lower chamber were then fixed with 95% alcohol for 15-20 min and stained with 0.5% eosin (Beijing Solarbio Science & Technology Co., Ltd.) for 10 min. The cells in 5 randomly selected

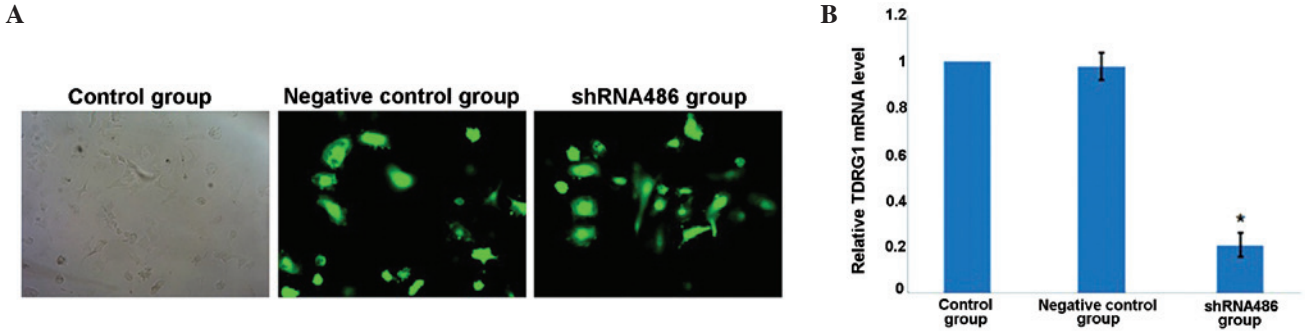

Figure 1. (A) The NTERA-2 cells in the control group did not demonstrate green fluorescence under the exciting wavelength. The transfection efficiency was ≥80% for the other two transfection groups. (B) Comparison of the relative TDRG1 mRNA expression levels between the three groups at 3 days post-transfection. The mRNA expression level of the TDRG1 gene in NTERA-2 cells transfected with psh486 was reduced by ~79% (*P<0.05 vs. the control group). TDGR1, testis developmental related gene 1; shRNA, short hairpin RNA.

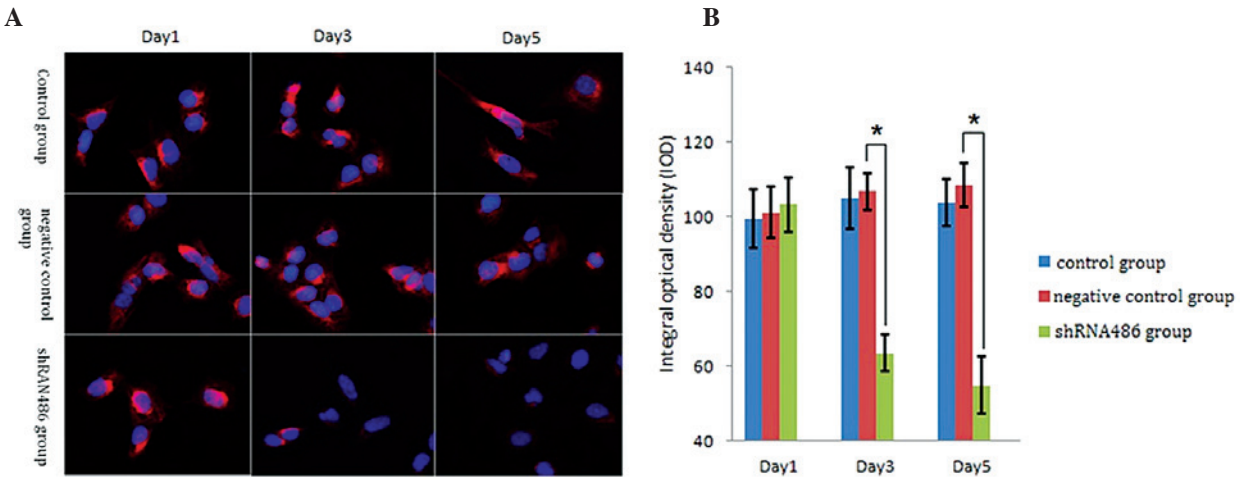

Figure 2. Immunofluorescence and the IOD value of the TDRG1 protein in the three groups. (A) The TDRG1 protein appears as red-orange fluorescence while the cell nucleus appears as blue. At days 3 and 5, the fluorescent signal of the TDRG1 protein in the shRNA486 group was reduced compared with day 1. (B) At days 3 and 5 post-transfection, the IOD value of the TDRG1 protein in the shRNA486 group was significantly reduced compared with the two control groups (*P<0.05). This result indicates that the expression of the TDRG1 protein in NTERA-2 cells was reduced following effective transfection. IOD, integral optical density; TDGR1, testis developmental related gene 1; shRNA, short hairpin RNA.

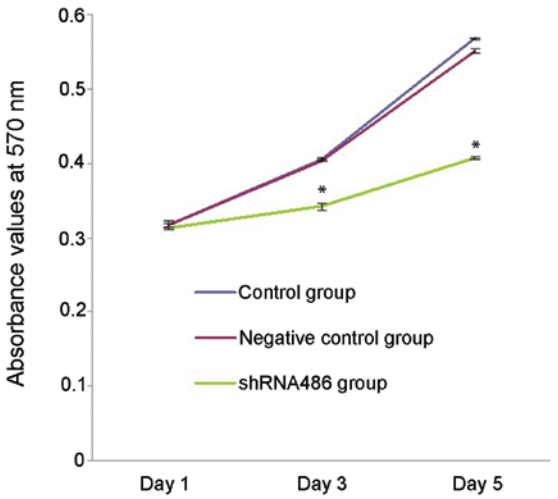

Figure 3. Detection of cell proliferation with MTT assay. A microplate reader was used to detect the optical density of each well at a wavelength of 570 nm at days 1, 3 or 5 post-transfection. The average value was calculated for each time point and group. The average absorbance values of shRNA486 group at days 3 and 5 were significantly lower than the other two groups (*P<0.001), indicating that the *in vitro* proliferation ability of this group was inhibited. shRNA, short hairpin RNA.

visual fields were counted under a light microscope (Olympus IX70; Olympus Corporation, Osaka, Japan), and the average value was recorded. This experiment was repeated 3 times.

Flow cytometric analysis. An annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) double-staining cell apoptosis test kit (KeyGene Inc., Nanjing, China) was used to detect the rate of apoptosis in each group. The cells were collected at 48 h following transfection and resuspended in the binding buffer containing annexin V-FITC and PI according to the manufacturer's instructions. All the samples were then analyzed using a FACScan flow cytometer (PT001374; BD Biosciences, San Jose, CA, USA) and the cells were classified as living cells, early apoptotic cells, advanced apoptotic cells and necrotic cells. The results were analyzed using BD FACSDiva software, version 6.1.3 (BD Biosciences) and the experiment was performed 3 times.

Statistical analysis. The results are expressed as the mean ± standard deviation. SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analyses

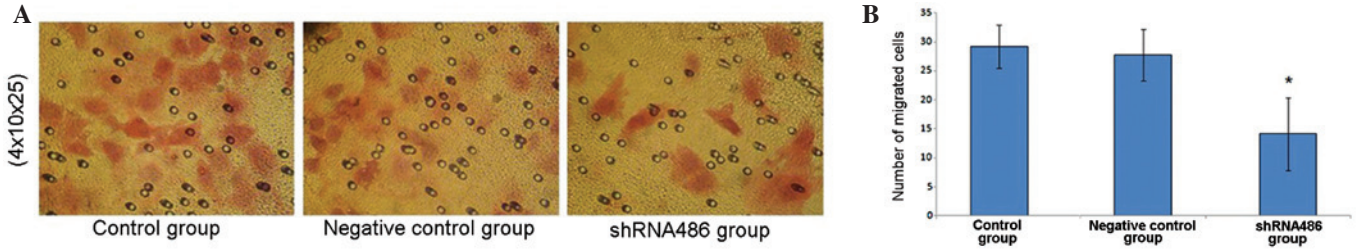

Figure 4. Analysis of cell invasion ability using a Transwell assay. (A) The cells penetrating the filter membrane were stained with eosin and appeared red under a light microscope. (B) Fewer cells penetrated the filter membrane in the shRNA486 group compared with the other two groups (*P<0.05). The *in vitro* invasion ability of the NTERA-2 cells transfected with the shRNA486 recombinant vector was inhibited by ≤49.1%. shRNA, short hairpin RNA.

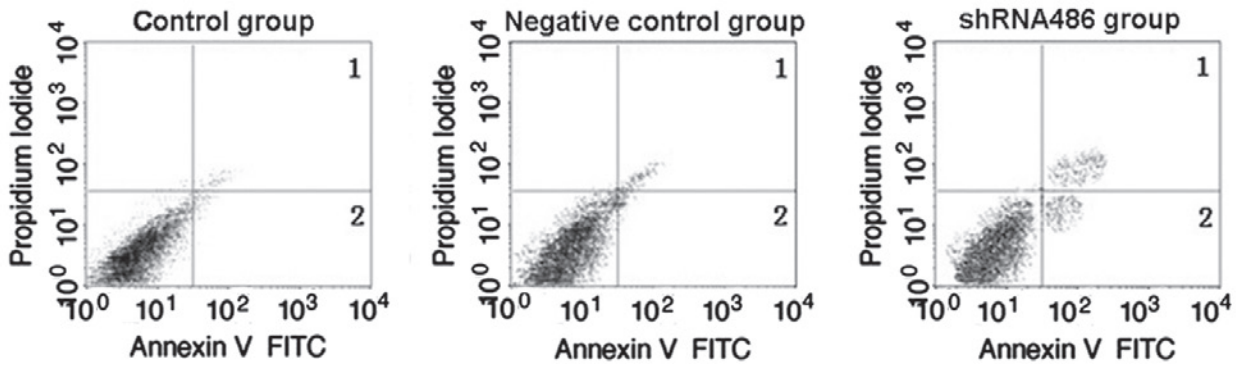

Figure 5. Measurement of cell apoptosis with annexin V/propidium iodide by flow cytometry. Quadrant 2 represents the proportion of early apoptotic cells, and quadrant 1 represents non-viable apoptotic cells and necrotic cells. The results indicated that silencing TDRG1 gene can induce apoptosis in NTERA-2 cells. TDRG1, testis developmental related gene 1; shRNA, short hairpin RNA; FITC, fluorescein isothiocyanate.

and the data were analyzed by analysis of variance. P≤0.05 was considered to indicate a statistically significant difference.

Results

TDRG1 expression levels were downregulated following transfection. At 3 days post-transfection, the expression levels of GFP were observed using the fluorescence microscope (Fig. 1A). Two types of recombinant vector were successfully transfected into NTERA-2 cells. The relative level of *TDRG1* mRNA expression in the negative control group was 0.99±0.04 (P>0.05 vs. the control group) on day 3, which was greater than that of the shRNA486 group (0.21±0.03, P<0.05 vs. the control group; Fig. 1B). Immunofluorescence detection was used to observe TDRG1 protein expression following shRNA transfection (Fig. 2A). At 1 day post-transfection, the IOD values of the TDRG1 protein did not significantly differ among the three groups (Fig. 2B; P>0.05): Control group, 99.41±7.76; negative control group, 101.19±8.18; and shRNA486 group, 103.31±6.17. However, at day 3 and 5 subsequent to transfection, the IOD value of the TDRG1 protein in the shRNA486 group was significantly lower compared with the other two groups (Fig. 2B; P<0.05). At 3 days post-transfection: Control group, 104.93±6.83; negative control group, 106.85±5.00; and shRNA486 group, 63.46±6.00. At 5 days post-transfection: Control group, 103.87±7.32; negative control group, 108.52±4.89; and shRNA486 group, 54.92±7.59. Notably, the IOD value of the TDRG1 protein in the negative control group was not significantly different compared with the control group (P>0.05).

TDRG1 silencing reduces the proliferation ability of NTERA-2 cells. At 1, 3 and 5 days post-transfection, the *in vitro* proliferation ability of NTERA-2 cells in each group was assessed using MTT assay (Fig. 3). The proliferation ability of NTERA-2 cells transfected with the psh486 was significantly reduced compared with the cells in the control group on day 3 (15.4% reduction, P<0.001) and day 5 (26.1% reduction, P<0.001) post-transfection. No significant differences in proliferation capacity were observed at days 1, 3 or 5 (P>0.05) between the control and negative control group.

TDRG1 silencing reduced the invasion ability of NTERA-2 cells. The cells that penetrated the filter membrane were stained with eosin and counted under the light microscope (magnification 4x10x25, Fig. 4A). The number of cells penetrating the filter membrane in the shRNA486 group was 14.13±6.24, compared with the numbers in the control and negative control groups, which were 29.20±3.70 and 27.73±4.47 cells, respectively (Fig. 4B). No significant difference was observed between the number of cells penetrating the filter membrane in the two control groups (P=0.728). There was a significant reduction in the number of cells penetrating the filter membrane in the shRNA486 group compared with the two control groups (P=0.010 vs. the control group and P=0.015 vs. the negative control group).

TDRG1 silencing induces the apoptosis of NTERA-2 cells. At 3 days post-transfection, the proportion of apoptotic cells in each group was detected using flow cytometry (Fig. 5). The proportions of early apoptotic cells present in each

group were as follows: shRNA486, 4.52±0.87%; control, 2.49±0.54%; and negative control, 2.58±0.88%. The proportions of total apoptotic cells present in each group were as follows: shRNA486, 9.72±1.37%; control, 5.97±0.96%; and negative control, 6.28±1.26%. The proportions of early apoptotic cells and total apoptotic cells were significantly increased in the shRNA486 group compared with the other two groups (P=0.019 vs. the negative control group and P=0.009 vs. the control group). No significant difference was demonstrated between the control and negative control groups (P>0.05).

Discussion

The majority of cases of testiculoma occur in young adults and the incidence is increasing; thus it is important to study its pathogenesis (13). The histological characteristics of testiculomas are complex. Germ cell tumors account for ~95% of cases, primarily consisting of seminoma and non-seminoma (4). Seminomas account for ~50% of TGCT cases and closely resemble intratubular germ cell neoplasia of an unspecified type (ITGCNU), which is the first step in the development of all TGCTs. Therefore, human seminoma cell lines may be the best cellular models for *in vitro* studies (14). However, with the exception of primary tumor samples, few tools are available to study the molecular pathogenesis of seminoma (15). To the best of our knowledge and according to the literature, only 3 cell lines (TCam-2, JKT-1 and SEM-1) originate from seminoma (16-18). Furthermore, the origin of these cell lines is not certain; therefore it remains controversial to describe these cell lines as seminoma cell lines (19,20). Taking these reasons into account, the present study used the recognized embryonal carcinoma NTERA-2 cell line as a cell model of TGCT (21,22).

Due to the poor differentiation characteristics and the multiple differentiation potential of ITGCNU, which is the common precursor of the majority of TGCT (23), the composition of TGCT is more closely associated with the choice of therapy compared with other urological neoplasms. Therefore, to determine the causes of differentiation in different types of tumor, studies at the gene and protein levels are particularly important. The onset and development of TGCT are complex processes involving multiple factors. Numerous proto-oncogenes and anti-oncogenes, including p53 and *c*-kit; multiple cell apoptosis genes, including Fas/Fas-L; the expression of the telomerase RNA component; and gene polymorphisms are all implicated in the process of TGCT development (24-28).

TDRG1 is a novel gene that was identified in a previous study by our research group through screening and cloning with digital differential display (7). Reverse transcription-polymerase chain reaction of multiple tissues demonstrated that *TDRG1* was expressed in human testes but not in other tissues, such as the heart, liver, brain, epididymis, lung, kidney and spleen. Immunohistochemical staining using a mouse anti-human TDRG1 monoclonal antibody (12) demonstrated that *TDRG1* was expressed solely in the testicular seminiferous tubules and spermatogenic cells. No expression was observed in the basal membrane or spermoblasts and a relatively higher expression level was observed in the testes of men between the ages of 15 and 34 years (7). Following analysis of TDRG1 protein expression levels under pathological states, including testiculoma, tuberculocele and testicular atrophy, using tissue microarray technology, another previous study demonstrated that the expression level of *TDRG1* in the tissues of TGCT, such as seminoma and teratoma, was significantly different compared with non-malignant human testis (8). Furthermore, significant differences were not observed in *TDRG1* expression levels between the tuberculocele and testicular atrophy groups (8). The characteristics of *TDRG1* expression levels in age, anatomical localization and pathological patterns indicated that TDRG1 may be associated with the oncogenesis, progression or transformation of TGCT.

RNAi degrades target mRNA by introducing exogenous or endogenous double stranded RNA into cells, resulting in inhibition of the corresponding gene (29). In studying the function of a target gene via upregulating or downregulating its expression, it is important to minimize alterations to the functions of other genes through off-target effects. shRNA-mediated RNAi technology is a targeted method for altering the expression of a specific gene (30). In our previously published work, four *TDRG1*-targeting shRNA recombinant plasmid vectors were successfully constructed using the pGPu6/GFP/Neo (10). psh486 was selected for use in the current study, as it was previously demonstrated the most successful at blocking the expression of the *TDRG1* gene. This recombinant vector was successfully transfected into NTERA-2 cells in the present study, and reduced the expression of *TDRG1* mRNA in the cells by 79% at 3 days post-transfection. The expression levels of the TDRG1 protein were also significantly reduced. Therefore, the *TDRG1* gene was silenced by psh486 in NTERA-2 cells with high efficiency. NTERA-2 cells display various biological characteristics of tumor cells. At 3 and 5 days following *TDRG1* gene silencing by psh486, the *in vitro* proliferation ability of NTERA-2 cells was inhibited by 15.4 and 26.1%, respectively. Furthermore, at 3 and 5 days, the early apoptotic potential and total apoptotic potential increased by 75 and 54.8%. In addition, the invasion process of NTERA-2 cells was simulated *in vitro* and demonstrated that the invasive ability of NTERA-2 cells was inhibited by 49.1% in cells transfected with psh486 compared with untransfected cells.

In conclusion, the present study indicated that the biological behavior of NTERA-2 cells may be closely associated with TDRG1, as the gene may promote the growth and invasion ability of these cells. The gene may be functional in TGCT and can be investigated further as a potential candidate gene in the development of TGCT. However the specific underlying molecular mechanism remains unclear and may involve interactions with other genes. Future studies are required to investigate these remaining questions in addition to the function of TDRG1 in cell lines derived from other testicular tumors.

Acknowledgements

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