G-patch domain containing 2, a gene highly expressed in testes, inhibits nuclear factor-κB and cell proliferation

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Abstract. G-patch domain containing 2 (GPATC2), a human gene that is highly expressed in the testes, was implicated as a novel cancer/testis antigen. The present study investigated GPATC2 expression in a number of human cell lines and rat tissues, and its potential biological function in 293T cells. Semi-quantitative reverse transcription-polymerase chain reaction analysis showed that GPATC2 was widely expressed in 15 human cell lines (representing different lineages) and in 11 different rat tissues, and that the GPATC2 mRNA relative expression level was significantly higher in the testis than it was in other tissues. 293T cells were transiently transfected with GPATC2-p enhanced green fluorescent protein (EGFP)-N1 or GPATC2-pEGFP-C3 and the nuclei were stained with 4',6'-diamidino-2-phenylindole. The results showed that GPATC2 is predominantly expressed in the nucleus of 293T cells. Overexpression of GPATC2 may inhibit transcription of the NF-κB reporter gene. The role of GPATC2 in proliferation was analyzed with cell counting kit-8, colony-forming efficiency and flow cytometry assays. The results indicated that over-expression of GPATC2 in 293T cells significantly inhibited cell proliferation by decreasing the number of cells in S phase. By contrast, GPATC2 knockdown by RNA interference exhibited the opposite effect, suggesting that GPATC2 may be involved in inhibiting G_1-S phase transition in 293T cells. In conclusion, these results provide novel insight into the breadth of expression of GPATC2 and its role in cell proliferation.

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Introduction

The mammalian testis serves two main functions: The synthesis of steroids and the production of spermatozoa. This second role is achieved through a process of transcriptional, translational and posttranslational regulation (1). A number of spermatogenesis-related genes, which are involved in specific phases of germ cell development, have been identified during the past few years, and certain genes have been shown to be important in spermatogenesis (2-3). However, spermatogenesis-derived factors involved in this process and the underlying molecular mechanisms remain largely obscure.

Cell division and proliferation, which are the most basic features of living organisms, are crucial in cell survival. Apoptosis, or programmed cell death, is the process by which excess or dysfunctional cells are eliminated, and is critical to maintain tissue homeostasis (4). Preserving the balance between cell proliferation and death is essential in sustaining differentiated tissue homeostasis and also forms a defense mechanism against pathogens (5). Nuclear factor (NF)-κB is a transcription factor that is important in regulating inflammation, immunity, cell proliferation and apoptosis (6-9).

Homo sapiens G-patch domain containing 2 (GPATC2; GenBank accession no. BC042193.1) also termed CT110, PPP1R30 and RP11-361K17.1, was first cloned by The National Institutes of Health Mammalian Gene Collection program in October, 2002, which is a multi-institutional effort to identify and sequence a cDNA clone containing a complete open reading frame for each human and mouse gene (10). It has been shown using northern blot analysis that the GPATC2 gene is highly expressed in the testes (11). The functions and mechanisms of action of the GPATC2 gene have not yet been elucidated. As no suitable male germ cell lines are currently available, a somatic cell line was used to investigate the function of GPATC2 in apoptosis, cell proliferation and the cell cycle.

The aim of the present study was to investigate the expression of GPATC2 in different human cell lines and rat tissues, and to determine the sub-cellular localization of the GPATC2 protein in 293T cells. In addition, the function of GPATC2 in 293T cell cycle progression was studied in order to provide novel insight into the breadth of expression of GPATC2 and its role in cell proliferation.

Materials and methods

Plasmid construction. The generation of vector pCMV-SPORT6-GPATC2 has been cloned by The Chinese National Human Genome Research Center (CHGB; Beijing, China), full-length GPATC2 the coding sequence was obtained by double enzyme digestion from pCMV-SPORT6-GPATC2 and cloned into the pcDNA3.1/myc-His (12) B vectors (pcDB; Invitrogen, Carlsbad, CA, USA), enhanced green fluorescent protein plasmid-N1 (pEGFP-N1) and pEGFP-C3, using the same enzyme restriction sites for *Eco*RI and *Bam*HI.

Preparation of small interfering RNAs (siRNA). The target sequence of the siRNA was 5'-GGAGCTGGTTCATGACCTT-3' for human GPATC2. Sense and antisense oligonucleotides with the internal loop were synthesized by Takara Bio Inc., (Tokyo, Japan). These were annealed and ligated into the *Bam*HI and *Hin*dIII sites of pSilencer 4.1-CMVneo (Ambion, Carlsbad, CA, USA) to construct the GPATC2-specific siRNA expression plasmid, according to the manufacturer's instructions. pSilencer 4.1-CMVneo expressing scrambled siRNAs (Ambion) was used as a control.

Immunofluorescence microscopy. 293T cells were transfected with si-GPATC2 or si-control and were grown on glass chamber slides. The cells were fixed using 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, permeabilized in 0.1% Triton X-100 for 30 min and then blocked with 0.5% bovine serum albumin in PBS for 30 min at room temperature (RT). After washing with PBS, the cells were incubated with polyclonal mouse anti-human GPATC2 antibody (H00055105-B01; Abnova, Walnut, CA, USA) for 2 h at 37˚C. After washing with PBS and Tween-20, the cells were incubated with rabbit anti-mouse fluorescein isothiocyanate-conjugated secondary antibody (F-4143; Sigma, St. Louis, MO, USA) and stained with 4',6-diamidino-2-phenylindole (DAPI; Roche, Basel, Switzerland). Images were visualized and captured using an Olympus microscope (Olympus, Tokyo, Japan).

Cell culture and transient transfection. H520, Hela, Hepg2, H1299, THP-1, Jurkas, U937, 786-O, HL60, A498, Caki, G401, MCF-7, T47d and 293T cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium or RPMI-1640 (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA) under 5% CO_2 at 37˚C. 293T cells seeded at a density of $1x10^4$ cells/well in 96-well plates or $3x10^5$ cells/well in 6-well plates and were transfected with empty vector pcDB or pcDB-GPATC2 using Vigorous transfection reagent (Vigorous Instruments Co., Ltd., Beijing, China), according to the manufacturer's instructions.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Male Sprague-Dawley rats were purchased from Peking University Laboratory Animal Center (Beijing, China). The study was approved by the Ethics Committee of Hebei United University (Tangshan, China). Rats were sacrificed by cervical dislocation. Appendix, liver, heart muscle, lung, small intestine, skeletal muscle, pancreas, brain, kidney, stomach and testis tissues were obtained from three rats of the same age. Total mRNA was extracted from all tissues and cell lines using the TRIzol RNA isolation protocol (Gibco-BRL). Samples (0.25 μg) were used for cDNA synthesis using the PrimeScript RT Master mix (Perfect Real Time) (Takara Bio Inc.). GPATC2 and GAPDH internal control transcripts were amplified by RT-PCR using the following primers: Forward: 5'-GCGCAGGCCGTCATCAAC-3' and reverse: 5'-TCCCACCAGGGCACAACTC-3' for human GPATC2; forward: 5'-GCCAGTATCCCGAGTTTAT-3' and reverse: 5'-GCCCGACTCCTTCTCATA-3' for rat GPATC2; and forward: 5'-AAGAAGGTGGTGAAGCAGGC-3' and reverse: 5'-TCCACCACCCTGTTGCTGTA-3' for GAPDH.

Subcellular localization. 293T cells seeded at a density of $1x10^4$ cells/well in 96-well plates were transfected with pEGFP-N1-GPATC2 or pEGFP-C3-GPATC2. After 24 h, cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min at RT, washed again with PBS and stained with DAPI (5 μg/ml in PBS) at RT for 5 min. Cells were visualized using an inverted fluorescence microscope (Nikon TE 2000-U, Nikon, Tokyo, Japan).

Dual-luciferase reporter assay. 293T cells were seeded into 96-well plates at $1x10^4$ cells/well. After 24 h, cells were co-transfected with 80 ng/well pcDB-GPATC2 or pcDB, 40 ng pNF-κB-Luc plasmid containing the firefly luciferase reporter gene (PathDetect, Stratagene, La Jolla, CA, USA) and 4 ng internal control pRL-TK plasmid containing the renilla luciferase gene (Promega Corporation, Madison, WI, USA). Each transfection was conducted in triplicate wells. After 24 h, cells were lysed in standard lysis buffer (Promega Corporation) and cell lysates were assayed for firefly and renilla luciferase activities using the dual-luciferase reporter (DLR) Assay system (Promega Corporation), according to the manufacturer's instructions. Light emission was measured using a GENios Pro reader (Tecan, Männedorf, Switzerland). Luciferase activity was normalized to renilla luciferase activity. Duplicate experiments were performed.

Cell counting kit-8 (CCK-8) assay. 293T cells transfected with pcDB or pcDB-GPATC2 were seeded into 96-well plates at a density of $2x10^3$ cells/well. At the indicated time point, the cell counting kit proliferation assay (Dojindo, Tokyo, Japan) was performed by adding 10 μl CCK-8 solution to each well and incubating at 37˚C for 2 h. Absorbance at 450 nm was then measured using a microplate plate reader (Synergy 2 Multi-Mode Microplate Reader, BioTek, Winooski, VT, USA).

Flow cytometry. 293T cells were synchronized by serum starvation for 12 h and then transfected with 2.5 μg pcDB or pcDB-GPATC2. Cells were trypsinized 48 h after GPATC2 transfection, washed twice with PBS and fixed with ice-cold 70% ethanol. Fixed cells were pelleted, washed and resuspended in PBS. Samples were then treated with 1 μl of 10 mg/ml DNase free RNase A (Promega Corporation) incubated at 37˚C for 30 min and then 50 μl of 300 mg/ml propidium iodide (Boehringer, Mannheim, Germany) containing

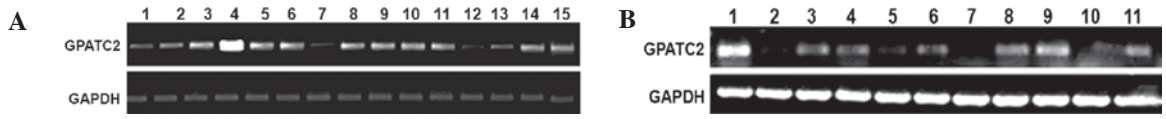

Figure 1. Expression profile of human GPATC2. (A) RT-PCR analysis of GPATC2 expression in cell lines. GAPDH was used as a control. Lane 1, H520 cells; lane 2, Hela cells; lane 3, 293T cells; lane 4, Hepg2 cells; lane 5, H1299 cells; lane 6, THP-1 cells; lane 7, Jurkas cells; lane 8, U937cells; lane 9, 786-O cells; lane 10, HL60 cells; lane 11, A498 cells; lane 12, Caki cells; lane 13, G401cells; lane 14, MCF-7 cells; and lane 15, T47d cells. (B) RT-PCR analysis of GPATC2 expression in normal rat tissues. GAPDH was used as a control. Lane 1, rat testis; lane 2, rat appendix; lane 3, rat liver; lane 4, rat heart muscle; lane 5, rat lung; lane 6, rat small intestine; lane 7, rat skeletal muscle; lane 8, rat pancreas; lane 9, rat brain; lane 10, rat kidney; and lane 11, rat stomach. GPATC2, G-patch domain containing 2; GAPDH, glyceraldehyde 3-phsophate dehydrogenase; RT-PCR, reverse transcription polymerase chain reaction.

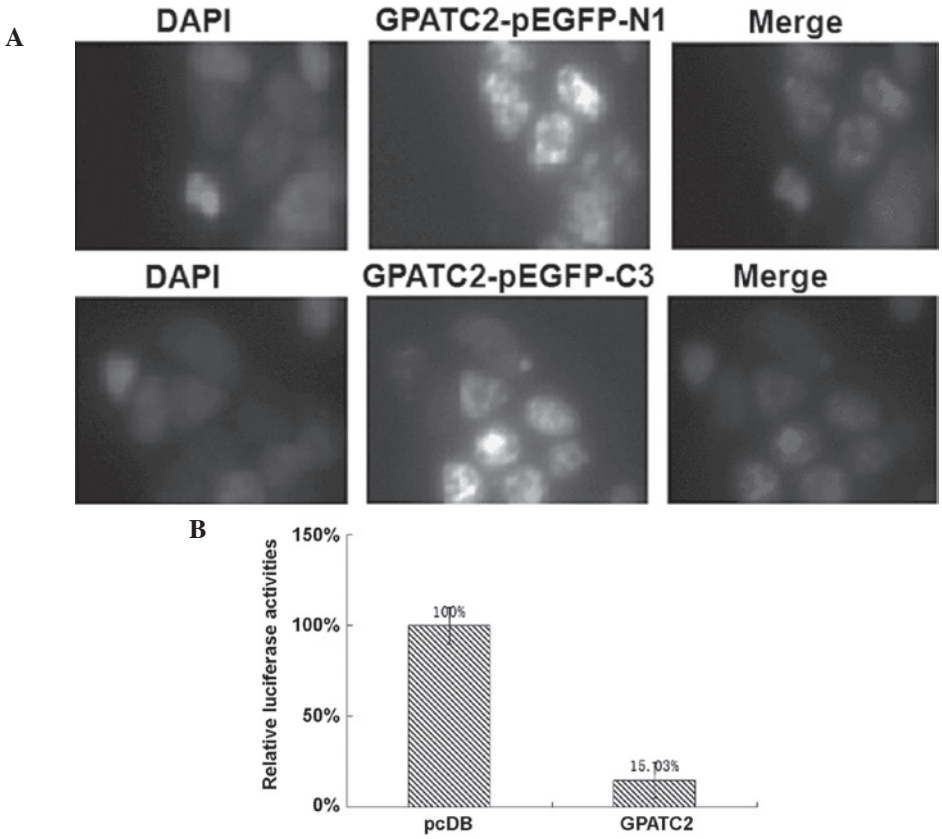

Figure 2. GPATC2 subcellular localization and inhibition of NF-κB in 293T cells. (A) Subcellular localization of GPATC2. 293T cells were transiently transfected with GPATC2-pEGFP-N1 or GPATC2-pEGFP-C3 and DAPI was used to stain nuclei. GFP and DAPI were visualized by fluorescence microscopy (magnification, x10). (B) GPATC2 inhibited NF-κB activation in 293T cells. 293T cells were transfected with pcDB-GPATC2 or empty vector pcDB along with pNF-κB-luc (80 ng). The DLR assay was performed using lysates from transfected cells. $^{*}P<0.05$ in an unpaired Student's t-test, compared with vector alone. GAPTC2, G-patch domain containing 2; NF-κB, nuclear factor-κB; pEGFP, ehanced green fluorescent protein plasmid; GFP, green fluorescent protein; DAPI, 4',6'-diamidino-2-phenylindole; DLR, dual-luciferase reporter.

Triton X-100 was added and cells were incubated in the dark for 30 min. Cell cycle data were obtained by flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA).

Results

Characterization and expression profile of the GPATC2 gene. The putative GPATC2 protein is comprised of 376 amino acids with a deduced molecular mass of ~43 kDa and a theoretical isoelectric point of 7.65. The putative GPATC2 protein has no transmembrane helices as predicted by the program TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/), and no other domains, including signal peptides, were found by the InterProScan program (http://www.ebi.ac.uk/Tools/InterProScan/). Human GPATC2 is located on chromosome 1q41. As shown in Fig. 1A, GPATC2 mRNA was widely expressed in the 15 human cell lines examined, which represented different lineages, as measured by RT-PCR. This technique was also performed to analyze GPATC2 mRNA expression in 11 different rat tissues, including appendix, liver, heart muscle, lung, small intestine, skeletal muscle, pancreas, brain, kidney, stomach and testis tissues. As shown in Fig. 1B, GPATC2 mRNA was expressed in a number of these tissues, although the GPATC2 mRNA relative expression level was significantly higher in the testis than it was in other tissues.

Over-expressed GPATC2 localizes to the nucleus and inhibits NF-κB. To determine the sub-cellular localization of GPATC2, GPATC2 cDNA was fused to the 3' end of the green fluorescent

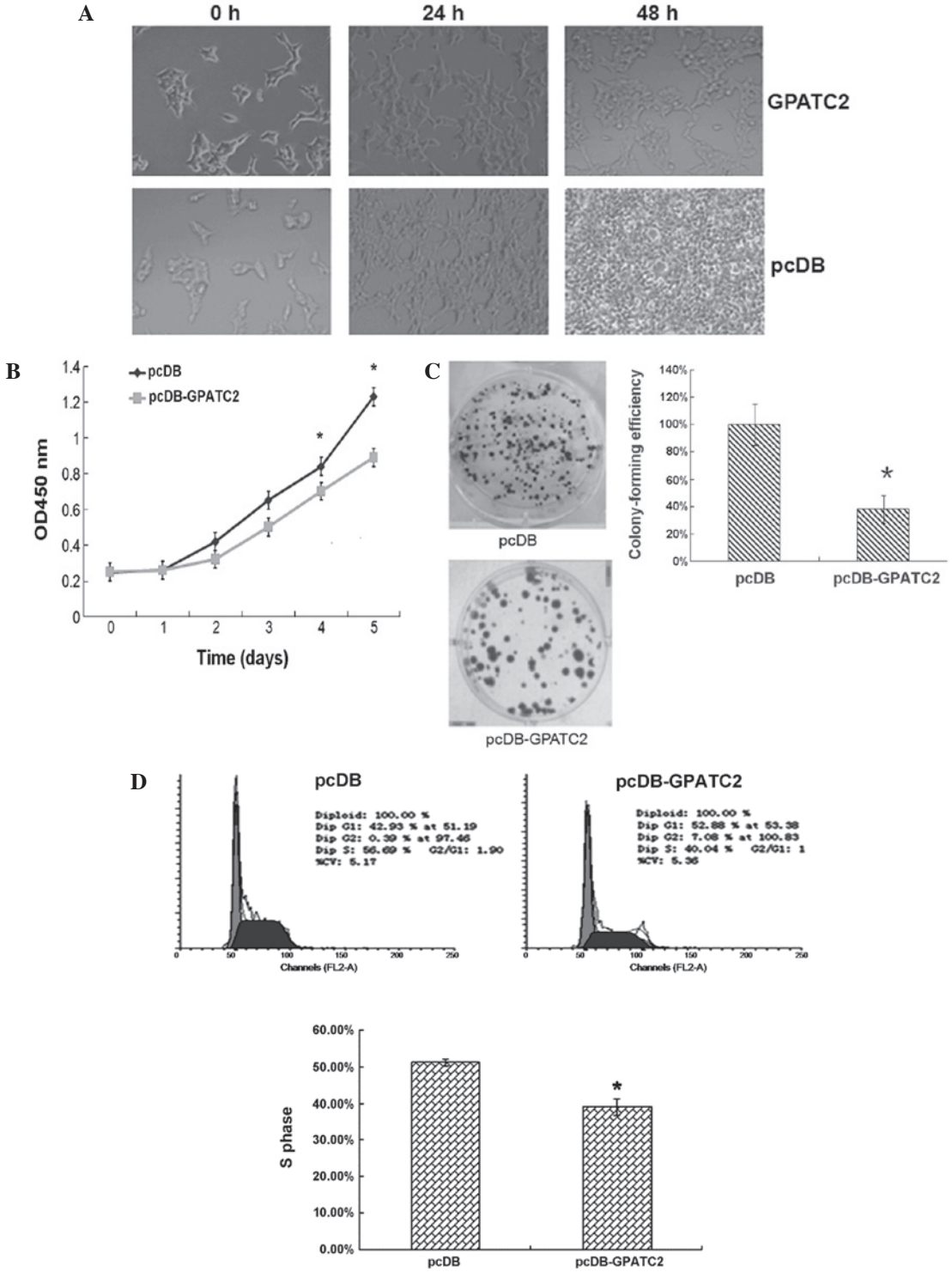

Figure 3. GPATC2 overexpression inhibits cell proliferation. (A) 293T cells transfected with pcDB-GPATC2 or pcDB (control vector) were observed under a microscope (magnification, x200). (B) 293T cells transfected with pcDB or pcDB-GPATC2 were assayed using CCK-8. The cells were collected and analyzed every 24 h and the absorbance was measured at 450 nm. (C) 293T cells were transfected with pcDB-GPATC2 or pcDB and CFE was analyzed after 14 days. The percentage CFE was calculated based on the total number of colonies in the control plates.(D) 293T cells transfected with pcDB-GPATC2 or pcDB were harvested, fixed, stained with propidium iodide and analyzed by flow cytometry, *P<0.05 in an unpaired Student's t-test compared with vector alone. GPATC2, G-patch domain containing 2; CCK-8, cell counting kit-8; CFE, colony forming efficiency; OD, optical density.

protein (GFP) cDNA in the pEGFP-C3 vector and to the 5' end of the GFP cDNA in the pEGFP-N1 vector. As shown in Fig. 2A, GPATC2-EGFP was predominantly expressed in the nucleus of 293T cells. The DLR assay system showed that GPATC2 over-expression in 293T cells inhibited the NF-κB reporter gene compared with the control group (Fig. 2B).

GPATC2 over-expression inhibits cell proliferation. As shown in Fig. 3A and B, cell numbers decreased significantly 24 h after GPATC2 over-expression was established compared with empty vector controls. As shown in Fig. 3C, colony-forming efficiency was 3.0-fold higher in controls cells than in cells over-expressing GPATC2 (Fig. 3C). This indicates a growth-inhibiting effect of

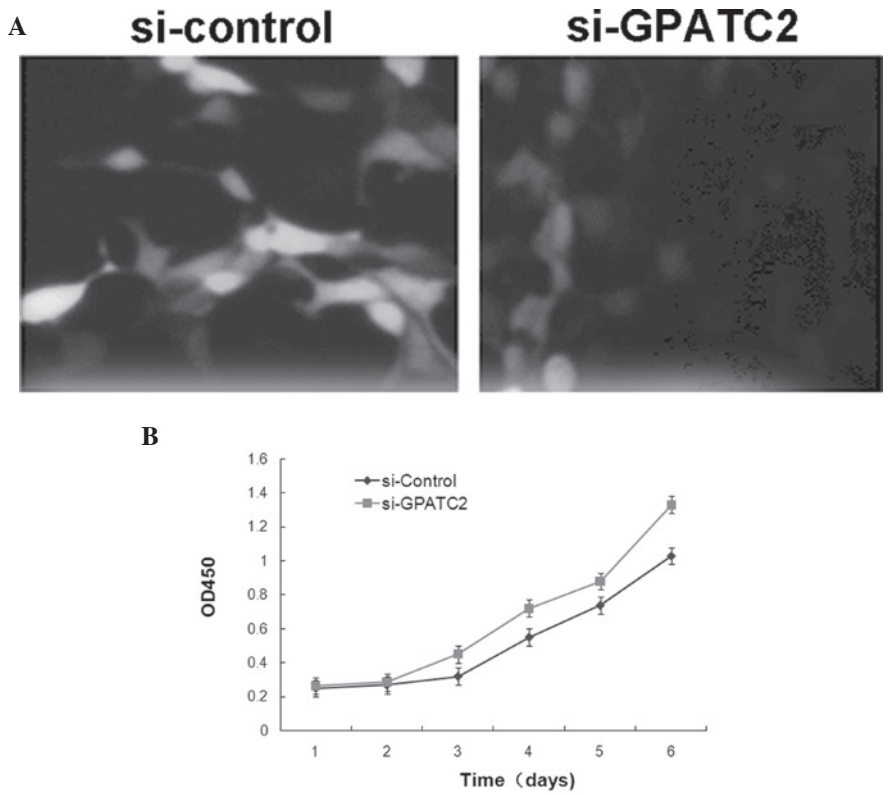

Figure 4. GPATC2 knockdown by RNA interference promotes cell proliferation. (A) The GPATC2-specific siRNA plasmid siRNA-GPATC2 was introduced into 293T cells. Control cells were treated with a scrambled siRNA. The efficiency of GPATC2 protein knockdown was examined by immunofluorescence (magnification, x200; stain, green fluorescent protein). (B) 293T cells transfected with si-control or siRNA-GPATC2 was assayed using CCK-8. Cells were collected and analyzed every 24 h and the absorbance was measured at 450 nm. *P<0.05 in an unpaired Student's t-test, compared with vector alone. GPATC2, G-patch domain containing 2; siRNA, small interfering RNA; CCK-8, cell counting kit-8.

GPATC2 on 293T cells. Furthermore, flow cytometry showed that a reduced proportion of 293T cells were in S phase 48 h after GPATC2 transfection, with 39.2% of cells over-expressing GPATC2 compared with 51.3% of controls in S phase (Fig. 3D). In addition, GPATC2 over-expression increased the proportion of cells in the G_0/G_1phase. These data suggest that GPATC2 over-expression significantly inhibits cell cycle G_1-S phase progression in 293T cells.

GPATC2 knockdown by RNA interference promotes cell proliferation. Based on the above results, it was hypothesized that knockdown of GPATC2 using RNA interference results in an increased number of cells in S phase. In order to test this hypothesis, an siRNA expression plasmid targeting GPATC2 or a scrambled control siRNA plasmid was transiently transfected into 293T cells. The reduction in GPATC2 expression was demonstrated by the use of immunofluorescence (Fig. 4A). As shown in Fig. 4B, in 293T cells depleted of GPATC2, cell numbers increased significantly after two days, compared with the empty vector-transfected cells. These data suggest that GPATC2 knockdown significantly promotes cell proliferation in 293T cells.

Discussion

GPATC2 exhibits high RNA expression in rat testis, an important organ involved in the synthesis of steroids and production of spermatozoa. Spermatogenesis is a complex biological process involving the mitotic proliferation of spermatogonia and the meiotic division of spermatocytes, followed by the morphogenic differentiation of spermatids to mature spermatozoa. This intricate process is reflected in the complex gene expression in the testis (13-14). This study shows that GPATC2 is a nuclear factor, which suggests that it may be important in spermatogenesis.

NF-κB is a generic term for a dimeric transcription factor formed by the heterodimerization or homodimerization of members of the Rel/NF-κB family (7). NF-κB-regulated genes are important for cell differentiation, embryonic development, the immune response and inflammation (15-17), as well as the development, progression and drug resistance of cancer cells (18,19). The present study demonstrated that GPATC2 inhibits the NF-κB pathway, suggesting that it may be an important regulator of a variety of cell functions. Furthermore, the results showed that GPATC2 inhibits 293T cell cycle progression by decreasing the number of cells in S phase, indicating that GPATC2 may be an important regulator of cell cycle progression. Lin *et al* (11) demonstrated that the interaction of GPATC2 protein with hPrp43, an RNA-dependent ATPase, significantly enhanced the ATPase activity of hPrp43 and induced a growth-promoting effect on mammalian cells. However, the mechanism through which GPATC2 inhibits 293T cell proliferation requires further elucidation.

In conclusion, GPATC2, which is highly expressed in the testis and is largely localized to the nucleus of 293T cells, is capable of inhibiting NF-κB and 293T

cell cycle progression *in vitro*. However, the mechanism of GPATC2 function may be far more complex *in vivo* and further investigations are required to elucidate the role and molecular mechanisms of GPATC2 in the process of spermatogenesis.

Acknowledgements

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