Microarray-based analysis of the gene expression profile in GC-1 spg cells transfected with spermatogenesis associated gene 12

YITING LIN, ZHIWEN LIU, XUANMING LIU, YUNSHENG ZHANG, ZHUOXIAN RONG and DAN LI

Department of Life Science, School of Biology, Hunan University, Changsha 410082, P.R. China

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Abstract. The unique differentiation mechanisms of spermatogenesis suggest the existence of cell type- and stage-specific molecules. Herein, a microarray-based approach was used to identify changes in the gene expression profile in mouse GC-1 spg germ cells transfected with spermatogenesis associated gene 12 (SPATA12) . One hundred and eighty-two upregulated genes and 104 downregulated genes with fold changes of ≥2 or ≤0.5 (P≤0.05) in expression were identified. Ten genes were selected for validation of the microarray results using quantitative RT-PCR. The real-time quantitative RT-PCR results were consistent with that of the microarray. The gene ontology (GO) terms for the biological functions of the differentially expressed genes induced by SPATA12 included binding activity and immune response. Biological pathway analysis identified several related pathways which are associated with immune responses, cell adhesion and the developmental process. In addition, we observed that SPATA12 may interact with the β-catenin signaling pathway and that SPATA12 may negatively regulate β-catenin signaling during spermatogenesis.

Introduction

Infertility affects approximately 15% of couples worldwide, and the male factor is at least partly responsible in approximately 50% of infertile couples (1). Genetic abnormalities, i.e. obstacles to spermatogenesis, are thought to account for 15-30% of male factor infertility (2). Spermatogenesis is the process by which male germ cells undergo a complex differentiation where morphological alterations lead to the formation of differentiated sperm. The unique differentiation mechanisms of spermatogenesis suggest the existence of cell type- and stage-specific molecules. With the advent of genetics and molecular biology, a variety of genes involved in spermatogenesis have been identified during the last decade (3-9).

Recently, we identified a novel protein referred to as spermatogenesis associated gene 12 (SPATA12) (10,11). In the testis, *SPATA12* is specifically expressed in spermatocytes, spermatids and spermatozoa, and may be involved in the development of testicular maturation. In contrast, the *SPATA12* gene is located on chromosome 3p14. Chromosomal abnormalities including homozygous deletions, loss of heterozygosity and expressional deficiencies in genes located at 3p14 have been frequently reported in many tumor types, suggesting that this locus is likely to contain tumour-suppressor genes (12,13). SPATA12 was also found to be absent in testicular germ cell tumors such as seminoma, yolk sac, teratoma and embryonal carcinoma. Flow cytometric analysis of SPATA12 in both mouse GC-1 spg germ cells and human HeLa cells indicates that the expression of the SPATA12 gene may delay G1 to S phase progression in the cell cycle. In addition, SPATA12 was shown to inhibit tumor cell colony formation. These findings suggest that SPATA12 could be an inhibitor suppressing cell proliferation in the process of germ cell development and in tumorigenesis. However, the transcriptional regulations of SPATA12 in spermatogenesis remain unclear.

The emerging technology of cDNA microarray hybridization offers the possibility of providing a rapid, high-throughput method for the efficient and accurate simultaneous expression measurement of thousands of genes (14-17). Studies based on microarray or expressed sequence tag analyses have been successfully used during spermatogenesis, and have aided in the understanding of the molecular mechanisms and genetic determinants of male infertility (18-20). Based on this background, we hypothesized that analysis of the gene expression profile induced by SPATA12 in GC-1 spg germ cells may contribute to an understanding of the function of SPATA12 and the possible pathways in which SPATA12 is involved during spermatogenesis.

Materials and methods

Cell line. The mouse GC-1 spg germ cell line (ATCC CRL-2053) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 μg/ml penicillin-

Correspondence to: Dr Dan Li, Department of Life Science, School of Biology, Hunan University, Changsha 410082, P.R. China
E-mail: lidanie@hotmail.com

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Table I. Primers used for real-time RT-PCR and RT-PCR experiments.

Gene name	Forward primer (5'→3')	Reverse primer (5'→3')	Product size (bp)
Gapdh1	GAAGGGTGGAGCCAAAAGG	TTGCTGACAATCTTGAGTGAGTTG	111
Ccl5	GCCCACGTCAAGGAGTATTTCT	TCTCTGGGTTGGCACACACTT	100
Fbxo39	AGCCTGAGGAGTTGCTATTTCAGT	ACGTTAACTTCTGCAGGGTGTTC	100
Wnt10a	CTTCAGCCGAGGTTTTCGAGA	CCGCAAGCCTTCAGTTTACC	108
Rtp4	CATCTTTGGGTGAGAAGGTGACT	GAGATCTGGGTGGTTTTACTTTGTG	120
Sp100	AGCTACAACCACAGTCCCCT	TCCTGTCCTTTTCCGTCTTCTAA	111
Zbp1	GACGGACAGACGTGGAAGATC	TTGACCGGATTGTGCTGACA	110
Gys1	CGCTGGAAGGGTGAGCTTT	GAAGTGGGCAACCACATACG	156
Mtss1	ATGGAGGCTGTGATCGAGAAG	TCCGGCTTTGTTTATGAAGTCTT	114
Selenbp1	AGCCAGGTCATCCACAGGTT	ACTTCGTGCTGTCCCCAAAG	100
Ndrg1	CACACAACATTTTGCTGTCTGC	GCCAACTGATCCATTGAGGGG	100
Cyclin B1	TGGCCTCACAAAGCACATGA	GCTGTGCCAGCGTGCTAATC	77
Cyclin D1	TAGGCCCTCAGCCTCACTC	CCACCCCTGGGATAAAGCAC	80
Cyclin E1	AATTGGGGCAATAGAGAAGAGGT	TGGAGCTTATAGACTTCGCACA	161
β-catenin	GGCAACCCTGAGGAAGAAGA	CACTGGTGACCCAAGCATTTT	597
Gapdh2	TTCAACGGCACAGTCAAGG	TGAAGTCGCAGGAGACAACC	694
SPATA12	CGCGGATCCATGTCCAGTTCTGCTCTGACT	CCCAAGCTTGCAGGATTATTATTGATTACAG	607

The primers of Gapdh1 were used for quantitative RT-PCR, while those for Gapdh2 were used for RT-PCR.

streptomycin, and was maintained in 5% CO_2 and a 95% humidified air atmosphere at 37˚C.

Transient transfection. Transfections were performed with Lipofectamine 2000 (Invitrogen). Cells were plated to 50-70% confluent culture in each well of a 6-well plate or a 60-mm dish 24 h before transfection. According to the manufacturer's instructions, the GC-1 spg cells were transfected with 4 μg pRevTRE plasmid or pRevTRE-SPATA12 plasmid, respectively.

RNA isolation. Total RNA was extracted with the TRIzol reagent (Invitrogen) according to the manufacturer's protocol, digested by RNase-free DNase (Fermentas), dissolved in diethyl pyrocarbonate-treated water, and stored at -80˚C prior to use. For quality control, RNA purity and integrity were evaluated by agrose gel electrophoresis and the OD260/OD280 ratio.

Microarray assay and data analysis. 32K Mouse Genome Array (CapitolBio Corporation, Beijing, China), covering 99% of the current assembly of the mouse genome and including 32,256 Oligo DNA with 70 mer length, was applied to investigate the possible changes in the mRNA level in GC-1 spg cells following SPATA12 gene transfection by comparison to control GC-1 spg cells. Total RNA was extracted using TRIzol reagent (Invitrogen) and then applied to synthesize Cy3- or Cy5-conjugated dUTP-labeled cDNA probe using the RNA Fluorescence Labeling Core kit (M-MLV version 2.0; Takara, Dalian, China) and following the manufacturer's instructions. Hybridization, scanning, and data extraction were conducted at CapitalBio Corporation. The number of genes affected by SPATA12 was determined using the scatter plots of control GC-1 spg cells vs. GC-1 spg cells transfected with SPATA12 (named GC-1 spg-SPATA12 cells). In addition, identified genes were also categorized specifically using biological process ontology terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathways using the software Molecule Annotation System 2.0 (http//:bioinfo.capitalbio.com/MAS/) (CapitolBio Corporation).

RT-PCR and SYBR-Green real-time PCR analysis. For cDNA synthesis, 2 μg of total RNA was reverse transcribed using M-MLV reverse transcriptase (Promega). Then PCR was performed in a 10 μl reaction volume containing 5.7 μl of nuclease-free water, 0.1 μl of Takara Taq (5 U/μl), 1 μl of 10X PCR buffer, 0.8 μl of dNTP mixture (2.5 mM), 2 μl of cDNA and 0.2 μl each of the 20 μM gene-specific primers. After initial denaturation for 10 min at 94˚C, 27-30 cycles of PCR were performed. Each cycle consisted of a denaturing period (30 sec at 94˚C), an annealing phase (55-60˚C), and an extension period (60 sec at 72˚C). After the last cycle, all samples were incubated for an additional 10 min at 72˚C. PCR products were separated by 1.5% agarose gel electrophoresis, and the DNA bands were stained with ethidium bromide. RT-PCR signals were normalized to the signals of the murine glyceraldehyde-3-phosphate dehydrogenase (gapdh) gene.

For quantitative RT-PCR, a SYBR-Green real-time RT-PCR protocol (Invitrogen) was applied using an MX3000 (Stratagene, USA) instrument. PCR was performed in a 10 μl reaction volume containing 2.2 μl of nuclease-free water, 5 μl of Mix, 2 μl of cDNA and 0.4 μl each of the 2.5 μM gene-specific primers. The PCR profile was 95˚C for 5 min followed by 94˚C for 30 sec, 58˚C for 20 sec, and 72˚C for 20 sec for 40 cycles, with a final extension at 72˚C for 10 min and storage at 4˚C. The level of gene mRNA was evaluated in automated analysis by MxPro. All the primers for RT-PCR are shown in Table I.

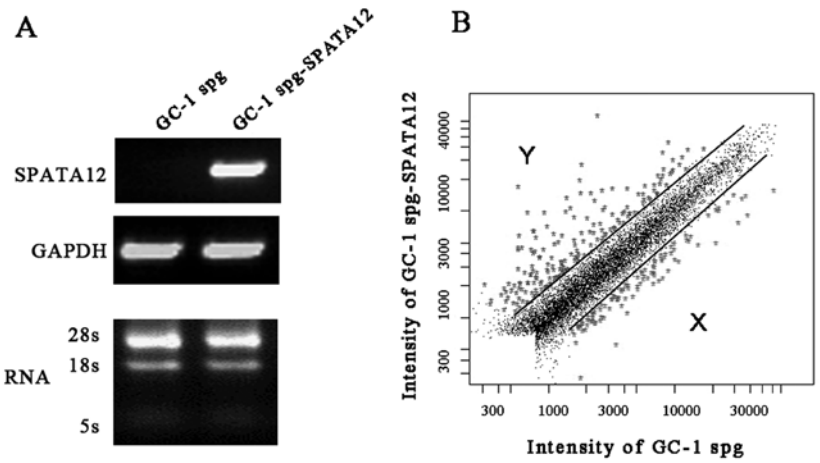

Figure 1. Global gene expression induced by SPATA12 in GC-1 spg cells. (A) The RT-PCR analysis of SPATA12 mRNA in GC-1 spg and GC-1 spg-SPATA12 cells. The quality of RNA used for the microarray analysis was assessed by agarose gel electrophoresis. (B) The log-log scatter-plot of the microarray results. Plots in region Y represent upregulated genes, while plots in region X represent downregulated genes in GC-1 spg following SPATA12 transfection.

Table II. Selected genes with differential expression between GC-1 spg and GC-1 spg-SPATA12 cells.

Term description	Symbol	Ratio	Gene name	Gene function
Upregulated genes	Ccl5	32.0033	Chemokine (C-C motif) ligand 5	Cytokine activity; chemokine activity; chemoattractant activity; immune regulation
	Rtp4	11.7258	Receptor transporter protein 4	Unknown
	Zbp1	8.7427	Z-DNA binding protein 1	Left-handed Z-DNA binding; RNA binding; double-stranded RNA adenosine deaminase activity; innate immune response
	Fbxo39	6.7244	F-box protein 39	Cancer/testis antigen
	Sp100	4.1610	SP100 nuclear antigen	Tumorigenesis; immunity; gene regulation
	Wnt10a	2.1549	Wingless related MMTV integration site 10a	Signal transducer activity; receptor binding; embryogenesis; carcinogenesis
Downregulated genes	Gys1	0.4613	Glycogen synthase 1 (muscle)	Catalytic activity; glycogen (starch) synthase activity; protein binding; transferase activity; transferase activity, transferring glycosyl groups
	Mtss1	0.3599	Metastasis suppressor 1	Unknown
	Ndrg1	0.2371	N-myc downstream regulated gene 1	Stress responses; hormone responses; cell growth; cell differentiation; p53-mediated caspase activation and apoptosis
	Selenbp1	0.1536	Selenium binding protein 1	Selenium binding; selenium-dependent role in ubiquitination/deubiquitination-mediated protein degradation

Statistical analysis. Statistical analysis was carried out using the Student's t-test. P-values ≤0.05 were considered to indicate statistically significant results.

Results

Global gene expression induced by SPATA12 in GC-1 spg cells. Global gene expression levels induced by SPATA12 in GC-1 spg cells from the microarray analysis are represented in Fig. 1. The differentially expressed genes with fold changes of ≥2 or ≤0.5 (P≤0.05) were analyzed using t-test and P-value and clustered with the software package Cluster 3.0. Our data showed that the expression of 286 out of 32,256 genes was altered after SPATA12 was expressed. Of these, 182 genes were upregulated and 104 genes were downregulated specifically.

Verification of the differentially expressed genes from the microarray data. To test the reliability of the microarray data, the expression patterns of 10 selected genes (including 6 upregulated genes and 4 downregulated genes) (Table II)

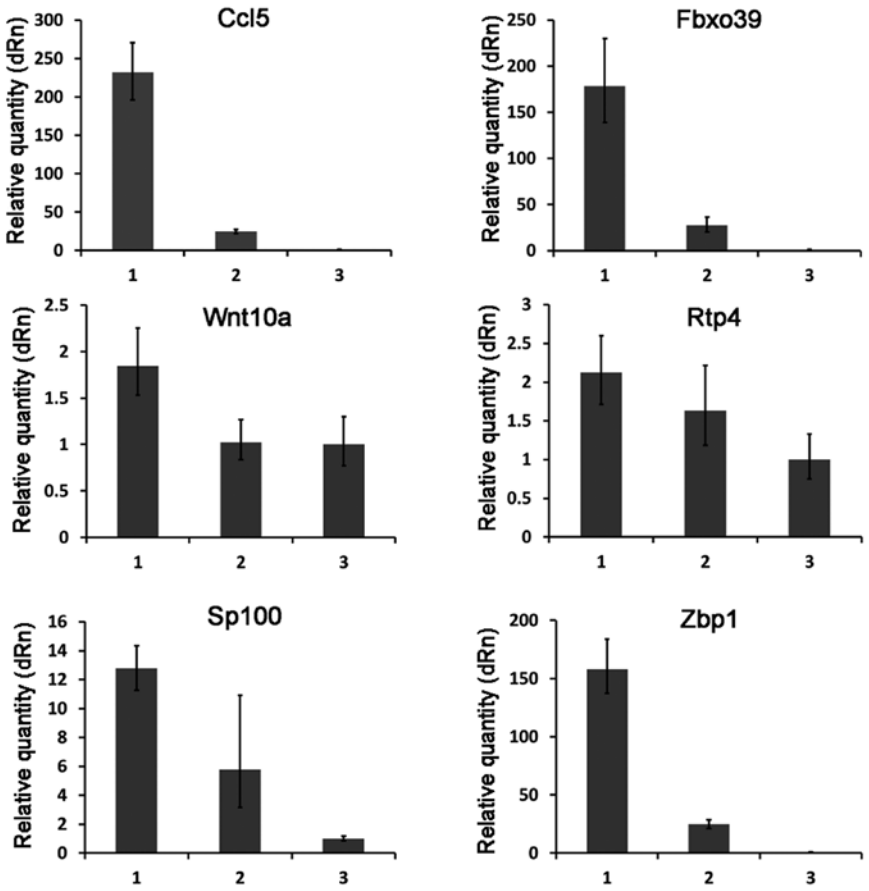

Figure 2. Validation of the expression of upregulated genes by real-time RT-PCR. Histogram: bar 1, GC-1 spg cells transfected with the pRevTRE-SPATA12 plasmid; bar 2, GC-1 spg cells transfected with the pRevTRE plasmid; bar 3, control GC-1 spg cells.

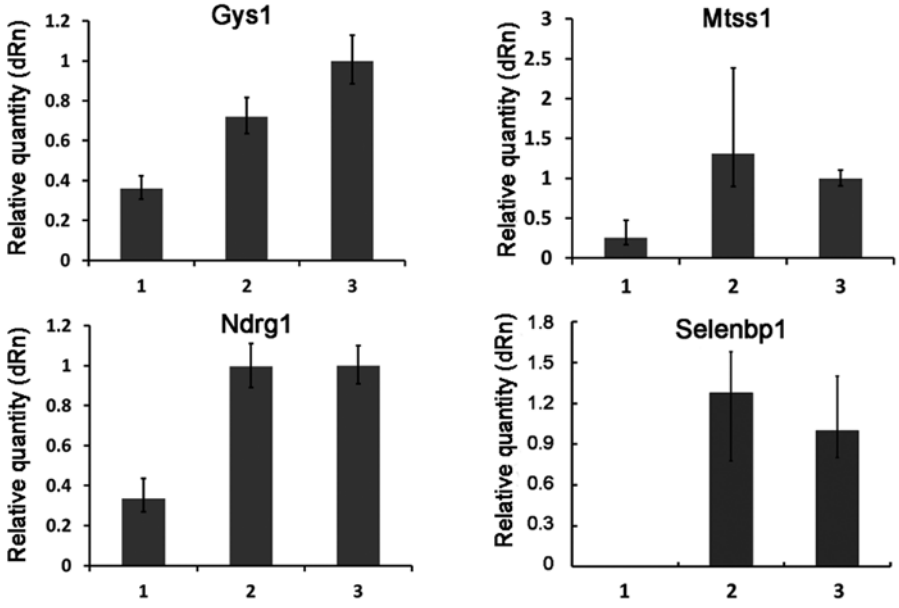

Figure 3. Validation of the expression of the downregulated genes by real-time RT-PCR. Histogram: bar 1, GC-1 spg cells transfected with the pRevTRE-SPATA12 plasmid; bar 2, GC-1 spg cells transfected with the pRevTRE plasmid; bar 3, control GC-1 spg cells.

were examined by quantitative real-time RT-PCR. The overall profile of gene expression by real-time RT-PCR analysis was similar to that revealed by the microarray data for all the selected genes (Figs. 2 and 3).

Gene ontology (GO) analysis of the microarray data. To gain insight into the potential functional consequences of the SPATA12-induced expression in GC-1 spg cells, GO analysis was applied to distribute genes into groups according to

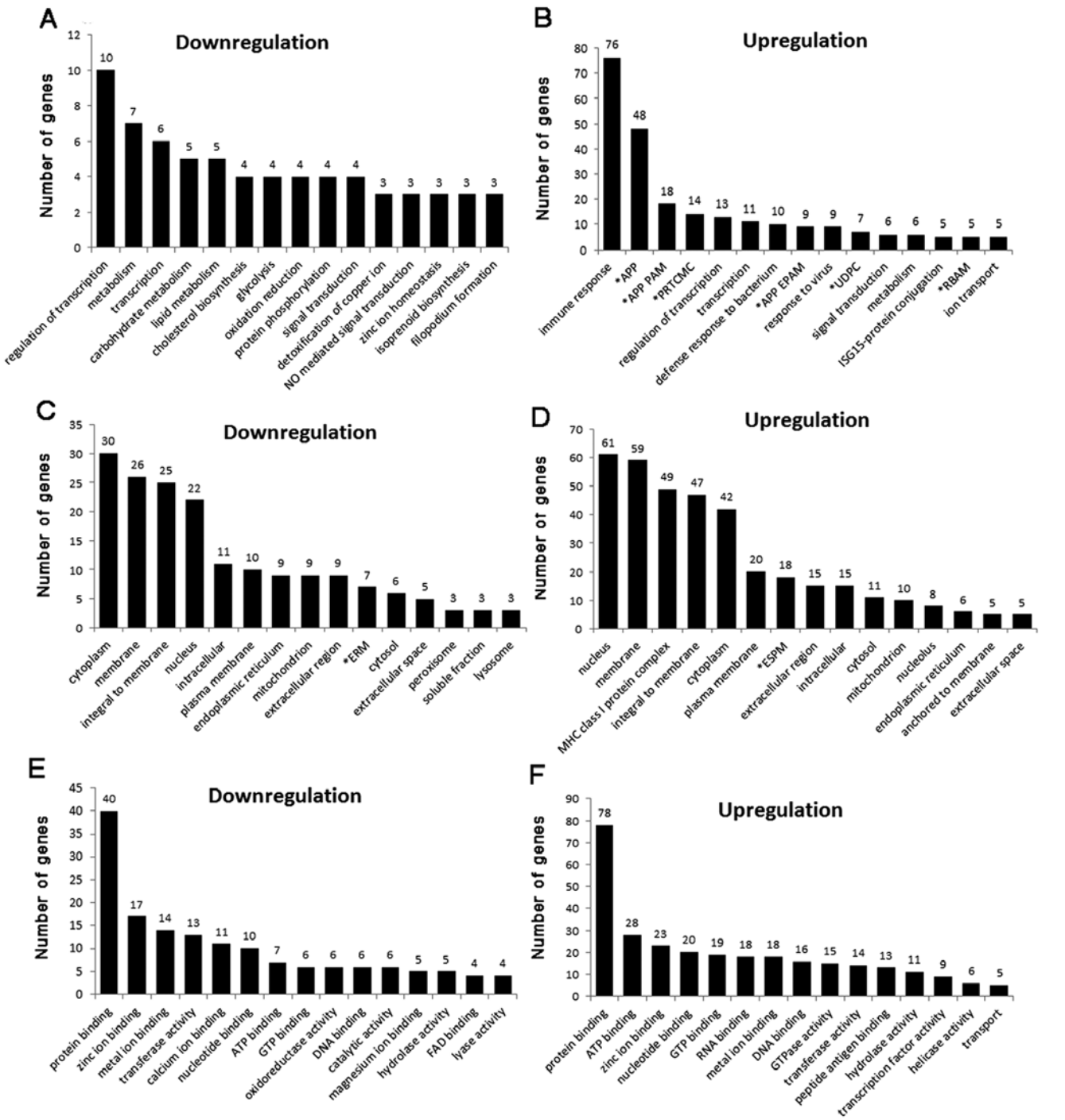

Figure 4. The number of SPATA12-induced genes in each of the (A and B) biological process, (C and D) cellular component and (E and F) molecular functional categories described in the Gene Ontology website. (A, C and E) Downregulated genes; (B, D and F) upregulated genes. *APP, antigen processing and presentation; *APP PAM, antigen processing and presentation of peptide antigen via MHC class I; *PRTCMC, positive regulation of T cell mediated cytotoxicity; *APP EPAM, antigen processing and presentation of exogenous peptide antigen via MHC class I; *UDPC, ubiquitin-dependent protein catabolism; *RBAM, response to bacterium associated molecule; *ERM, endoplasmic reticulum membrane; *ESPM, external side of plasma membrane.

biological process, molecular function and cellular component, respectively.

Functional analysis using GO of the biological process group revealed that genes related to immune response, antigen processing and presentation, positive regulation of T cell-mediated cytotoxicity, transcription regulation, and defense response to bacterium were upregulated in the SPATA12-transfected GC-1 spg cells (Fig. 4A and B). This suggests that based on the GO terms these upregulated genes combined with SPATA12 may play important roles in the physiology of the immune response. In contrast, genes related to carbohydrate metabolism, lipid metabolism, cholesterol biosynthesis and glycolysis were downregulated in the SPATA12-transfected cells, which indicates that based on these GO terms these genes affected by SPATA12 may be involved in metabolic processes.

Table III. Selected pathways related with SPATA12 identified by biological pathway analysis.

Pathway name	Total	Gene (ratio)
Antigen processing and presentation	12	H2-Q10 (2.36), H2-Q7 (2.79), H2-T9 (2.49), H2-T3 (2.01), H2-Q6 (2.02), Tap1 (2.3442), Psme2 (2.4576), H2-K1 (2.28), H2-Q1 (2.92), Psme2b-ps (2.4576), B2m (2.5449), H2-T23 (2.24)
Cell adhesion molecules (CAMs)	9	H2-Q10 (2.36), H2-Q7 (2.79), H2-T9 (2.49), H2-T3 (2.01), H2-Q6 (2.02), Itgb7 (2.73), H2-K1 (2.28), H2-Q1 (2.92), H2-T23 (2.24)
Type I diabetes mellitus	8	H2-T23 (2.24), H2-Q1 (2.92), H2-K1 (2.28), H2-Q6 (2.02), H2-T3 (2.01), H2-T9 (2.49), H2-Q7 (2.79), H2-Q10 (2.36)
Toll-like receptor signaling pathway	5	Myd88 (2.0637), Cxcl10 (5.5093), Stat1 (5.04), Cd14 (2.0947), Ccl5 (32.0033)
MAPK signaling pathway	4	Ddit3 (2.0538), Cd14 (2.0947), Dusp6 (2.9646), Map4k2 (0.4768)
Biosynthesis of steroids	3	Sqle (0.4314), Pmvk (0.4707), Fdft1 (0.4985)
ECM-receptor interaction	3	Col6a1 (0.4919), Col5a3 (0.4825), Itgb7 (2.7347)
Jak-STAT signaling pathway	3	Isgf3g (3.57), Stat1 (5.04), Stat2 (2.55)
Cytokine-cytokine receptor interaction	3	Ccl5 (32.0033), Vegfa (0.4651), Cxcl10 (5.5093)
Natural killer cell mediated cytotoxicity	2	H2-T23 (2.24), H2-K1 (2.28)
T cell receptor signaling pathway	1	Pdk1 (0.4713)
Wnt signaling pathway	1	Wnt10a (2.15)

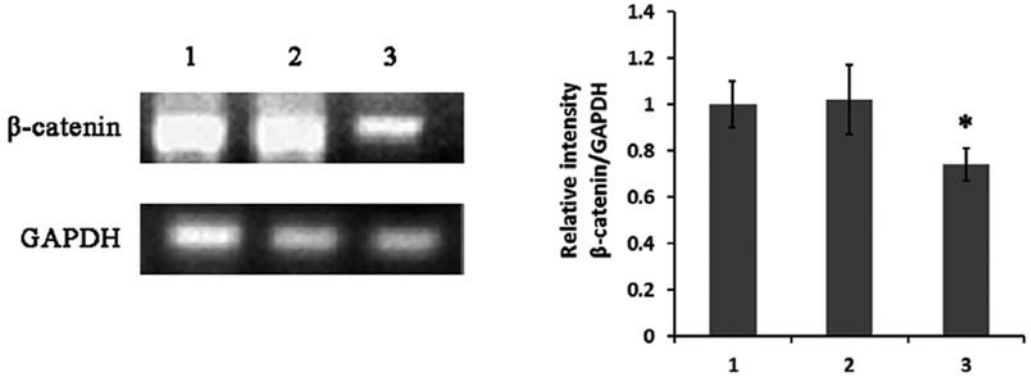

Figure 5. Expression of *β-catenin* in GC-1 spg cells transfected with SPATA12. Lane 1, control GC-1 spg cells; lane 2, GC-1 spg cells transfected with the pRevTRE plasmid; lane 3, GC-1 spg cells transfected with the pRevTRE-SPATA12 plasmid. Data are means ± SD (n=4). *P<0.05 vs. control (group 1) by t-test.

In the cellular component group (Fig. 4C and D), GO analysis showed that the major differentially expressed genes, including 61 upregulated and 22 downregulated genes, were located in the nucleus. These data indicate that SPATA12 may play roles in the nucleus through interacting with genes associated in these GO categories. This observation was consistent with our previous bioinformatics analysis report, which predicted that SPATA12 probably functions as a testis-specific nuclear protein involved in spermatogenesis (21).

We also noted that these genes (molecular function group) (Fig. 4E and F), either upregulated or downregulated in SPATA12-transfected cells, were mostly involved in protein binding, ATP/GTP binding, zinc ion binding or DNA/RNA binding, indicating that the function of SPATA12 may be related with the activity of 'binding'.

Pathway analysis and the expression change in β-catenin signaling induced by SPATA12. The analysis of the differential expression of genes led us to wonder whether these genes may represent related pathways in which SPATA12 is involved. Thus, biological pathway analysis was applied to characterize the unique gene networks associated with SPATA12 in germ cells. Most genes associated with immune-related pathways such as antigen processing and presentation, cell adhesion, T cell receptor signaling pathway, and development-related pathways such as MAPK, Jak-STAT and Wnt, were significantly overexpressed which provides evidence that both immune responses and developmental processes may be associated with various functions of SPATA12 (Table III). Changes in the expression of Wnt signaling-related genes such as β-catenin supported this possibility. Semi-quantitative RT-PCR results showed that the

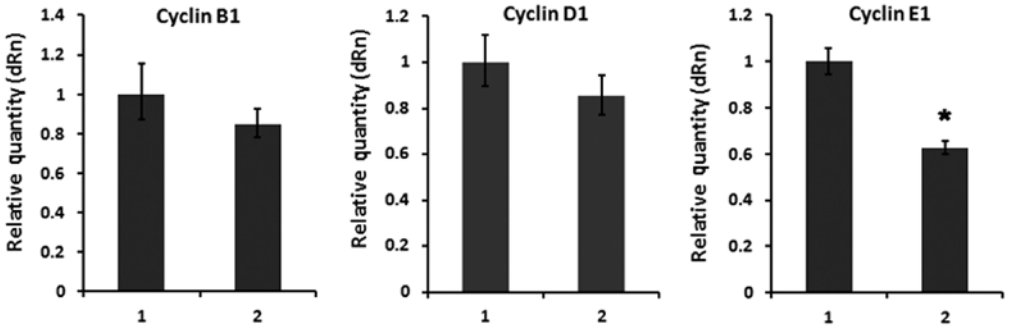

Figure 6. Expression of cell cycle-related genes in the GC-1 spg cells transfected with SPATA12. Histogram: bar 1, GC-1 spg cells transfected with the pRevTRE plasmid; bar 2, GC-1 spg cells transfected with the pRevTRE-SPATA12 plasmid. Data are means ± SD (n=4). *P<0.05 vs. control (group 1) by t-test.

expression of *β-catenin* was obviously downregulated (Fig. 5) and its downstream target cell cycle gene *cyclin E1* was strongly decreased (Fig. 6) in GC-1 spg cells transfected with SPATA12.

Discussion

High-density cDNA microarrays provide an important tool to study the global patterns of gene expression. For example, it has been used to understand interactions in a given cell, tissue and organism under normal and diseased states (22,23), or it has been used for gene discovery and function (24,25). Spermatogenesis is a complex process of cell development and differentiation that requires the highly regulated expression of multiple genes (26). Characterization and functional analysis of new testis-specific genes related to spermatogenesis is of momentous physiological and pathological significance in order to understand the molecular mechanisms of spermatogenesis. Herein, cDNA microarray was used to identify the upregulated or downregulated genes affected by SPATA12 and obtain a global overview on the expression patterns of these genes, aimed at acquiring a further understanding of the function of SPATA12 and the possible pathways in which SPATA12 is involved.

We identified 182 upregulated genes and 104 downregulated genes with a fold change of ≥2 or ≤0.5 (P≤0.05) in expression. Through quantitative real-time RT-PCR, we confirmed the expression of 10 (Ccl5, Fbxo39, Wnt10a, Rtp4, Sp100, Zbp1, Gys1, Mtss1, Selenbp1 and Ndrg1) of the differentially expressed genes. These genes along with their GO categories identified as being differentially expressed following induction by SPATA12 may be of significant biological interest.

Gene ontology studies provide biologically meaningful information regarding genes including the cellular location, molecular function and biological process. Fig. 4 lists each GO category and the specific number of genes in their respective category that were differentially expressed following induction by SPATA12. Biological pathway analysis identified several related pathways such as antigen processing and presentation, cell adhesion, MAPK, Jak-STAT and Wnt. The differential expression of these signaling pathway-related genes may also show that SPATA12 is associated with immune responses in germ cell development.

Alterations in the expression of Wnt/β-catenin signaling-related genes support this possibility. Wnt/β-catenin signaling is one of the most important developmental signaling pathways that control cell fate decisions and tissue patterning during early and late embryonic development. β-catenin is a key component of the Wnt/β-catenin signaling pathway (27-30) and was previously reported to be expressed in the plasma membrane and cytoplasm of germ cells during testis development. Suppression of Wnt/β-catenin signaling is necessary for the normal development of primordial germ cells since stabilization of β-catenin in germ cells was found to delay cell cycle progression resulting in germ cell deficiency (31). Here, we showed that expression of SPATA12 by transfection downregulated *β-catenin* in GC-1 spg cells. Studies in the literature reported that the reduction in β-catenin level was accompanied by inhibition of its transactivation potential and downregulation of downstream target genes, such as *Myc*, *cyclin D1* and *cyclin E1* (28,32,33). In the present study, our data suggest that SPATA12 negatively regulates cell cycle-related gene *cyclin E1*, which indicates that *SPATA12* inhibits cell proliferation via downregulation of *β-catenin* in GC-1 spg cells. These results were shown to be of particular relevance between SPATA12 and the β-catenin signaling pathway.

Taken together, the present study demonstrated alterations in the gene expression profile of GC-1 spg cells transfected with SPATA12. The functional classification of these genes and their expression profiles provide useful information to understand the transcriptional regulation of SPATA12. A number of genes, GO categories and biological pathways such as immune responses may be associated with the function of SPATA12. Moreover, our study showed that SPATA12 may interact with the β-catenin signaling pathway and SPATA12 could negatively regulate β-catenin signaling during spermatogenesis.

Acknowledgements

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