

# Screening for miRNAs and their potential targets in response to TGF- $\beta$ 1 based on miRNA microarray and comparative proteomics analyses in a mouse GC-1 spg germ cell line

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**Abstract.** Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is a member of the TGF- $\beta$  superfamily that performs a number of cellular functions and shows differential activity at different testicular developmental stages. In the present study, we investigated the effects of exogenous TGF- $\beta$ 1 on global microRNA (miRNA or miR) expression profiles by miRNA microarray analysis and the alterations in protein profiles by two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS) in a mouse GC-1 spg germ cell line. A total of 24 differentially expressed miRNAs, including 7 upregulated and 17 down-regulated miRNAs were identified. The results obtained by the RT-qPCR analysis of 10 selected differentially expressed miRNAs were in accordance with those obtained by miRNA microarray analysis. In addition, 11 differentially expressed proteins, including 3 upregulated and 8 downregulated proteins were identified through MS-based comparative proteomics analysis. Bioinformatics analysis predicted that peptidyl-prolyl isomerase A (PPIA) and nucleoside diphosphate kinase B (NDKB) are targets of miR-149 and miR-199a-3p, respectively in response to the stimulation of mouse GC-1 spg germ cells with TGF- $\beta$ 1. RT-qPCR revealed that the expression levels of these miRNAs showed an opposite trend in response to stimulation with TGF- $\beta$ 1. In conclusion, we identified some important miRNAs and proteins as possible targets involved in TGF- $\beta$ 1 signaling. Our data suggest the existence of a TGF- $\beta$ 1-miR-149-PPIA or TGF- $\beta$ 1-miR-199a-3p-NDKB pathway in GC-1 spg cells. Further studies are warranted to ascertain the role of these miRNAs in spermatogenesis.

## Introduction

Male infertility is a major factor in the inability of a couple to conceive. The most common cause of male infertility is disorders affecting spermatogenesis, which is a complex process strictly regulated by the cooperation of genetic factors, hormones and cytokines. Testicular cytokines and growth factors, such as interleukin (IL)-1, tumor necrosis factor (TNF), leukemia inhibitory factor (LIF), stem cell factor (SCF) and transforming growth factor (TGF) have been shown to affect both germ cell proliferation and testicular function (1,7). Under pathological conditions, the levels of cytokines are altered and negatively affect spermatogenesis. Thus, the expression levels and the regulatory pathway of cytokines in the testis should be taken into consideration in the development of therapeutic strategies for male infertility (1-3).

TGF- $\beta$ 1 is a member of the TGF- $\beta$  superfamily that controls proliferation, differentiation, embryonic development, angiogenesis and other functions in various cell types (4). It has been found that the expression levels of TGF- $\beta$ s vary at different stages of testicular development. They induce and maintain the specification of the germ cell lineage in the fetal testis, and regulate spermatogonial differentiation in the adult testis (5-7). Although studies on the TGF- $\beta$  signaling pathway have mainly focused on the ligand-receptor interaction and the specific biological effects at a cellular and/or molecular level (8), there also exist unknown cross-talks between TGF- $\beta$ s and other different signaling molecules, such as microRNAs (miRNAs or miRs), which are small non-coding RNAs that post-transcriptionally regulate gene expression by targeting mRNAs for translational repression and degradation (9).

Increasing evidence indicates that miRNAs are likely to be critically involved in the majority of biological processes, including mammalian germ-cell development (10). For instance, in Dicer-deleted testis, spermatogenesis is retarded at an early stage of proliferation and/or early differentiation (11). Recent data have indicated that the expression levels of miR-141, miR-200a, miR-200c and miR-323 in mice are downregulated gradually in both male and female germ cells throughout their development, suggesting that miRNAs are involved in translational repression during spermatogenesis (12). On the other hand, miRNAs have been found to target the TGF- $\beta$ 1 superfamily receptors, Smads or multiple components of the TGF- $\beta$ 1

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signaling pathway, and thereby also affect TGF- $\beta$ 1-regulated physiological or pathological processes, indicating that miRNAs are involved in the cellular response to TGF- $\beta$ 1 signaling (13). However, to the best of our knowledge, no systemic studies on the changes that occur in the expression levels of miRNAs induced by TGF- $\beta$ 1 signaling in mouse male germ cells have been published to date. Thus, in the present study, we investigated the effects of TGF- $\beta$ 1 on global miRNA and protein expression profiles in the mouse GC-1 spg germ cell line. In addition, we aimed to elucidate the association between these miRNAs and proteins and TGF- $\beta$ 1 signaling.

## Materials and methods

**Cell line.** Mouse GC-1 spg germ cells (CRL-2053; obtained from ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100  $\mu$ g/ml penicillin-streptomycin, and maintained in an atmosphere of 5% CO<sub>2</sub> and 95% humidified air at 37°C.

**Treatment with TGF- $\beta$ 1.** The cells (60% confluent) were maintained in serum-free DMEM for 24 h prior to stimulation with TGF- $\beta$ 1 (PeproTech, Rocky Hill, NJ, USA). Subsequently, the cells were stimulated with 5 or 10 ng/ml of TGF- $\beta$ 1 in DMEM containing 1% FBS for 48 h.

**RNA isolation.** Total RNA was extracted using the RNAiso reagent (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions, digested by RNase-free DNase (Fermentas, Burlington, ON, Canada), dissolved in diethylpyrocarbonate-treated water, and stored at -80°C until use. For quality control, RNA purity and integrity were evaluated by agarose gel electrophoresis and the OD260/OD280 ratio, respectively.

**miRNA microarray and data analysis.** The Affymetrix GeneChip miRNA 3.0 microarray (CapitalBio Corp., Beijing, China) covering the miRNAs from Sanger miRBase v17.0 ([www.mirbase.org](http://www.mirbase.org)), snoRNAs and scaRNAs from snoRNABase ([www.snorna.biotoul.fr/coordinates.php](http://www.snorna.biotoul.fr/coordinates.php)) and the Ensembl Archive ([www.ensembl.org/biomart/martview](http://www.ensembl.org/biomart/martview)) was applied to investigate the possible changes in miRNA expression in the GC-1 spg cells treated with different doses of TGF- $\beta$ 1 in comparison to the control (untreated) GC-1 spg cells. Total RNA was labeled with the FlashTag™ Biotin RNA Labeling kit following the manufacturer's instructions. Subsequently, hybridization, scanning and data extraction were carried out at CapitalBio Corp.. The number of miRNAs affected by TGF- $\beta$ 1 was determined using the scatter plots of the control GC-1 spg cells versus the GC-1 spg cells treated with TGF- $\beta$ 1. Finally, the images were gridded and analyzed using ImaGene 7.0 software (BioDiscovery Inc., Hawthorne, CA, USA).

**Reverse transcription-quantitative (real-time) PCR (RT-qPCR).** For the analysis of miRNA expression, RT-qPCR was performed. cDNA was made from enriched miRNA using miRNA PrimeScript RT Enzyme Mix (Takara Bio, Inc.). The SYBR-Green real-time PCR protocol (Takara Bio, Inc.) was then applied by using a MX3000 (Stratagene, La Jolla, CA, USA) instrument. PCR was performed in a 10- $\mu$ l reaction

volume containing 3  $\mu$ l of nuclease-free water, 5  $\mu$ l of SYBR Premix Ex Taq II, 0.2  $\mu$ l ROX Reference Dye II (both from Takara Bio, Inc.), 1  $\mu$ l of cDNA and 0.4  $\mu$ l each of the 10  $\mu$ M gene-specific primers. Following initial denaturation for 20 sec at 95°C, 39-45 cycles of PCR were performed. Each cycle consisted of a denaturation period (5 sec at 95°C), an annealing phase (30 sec at 55°C) and an extension period (30 sec at 72°C). U6 RNA was used as an endogenous control. miRNA primers were provided by Takara Bio, Inc.

**Preparation of protein samples and two-dimensional gel electrophoresis (2-DE).** In brief, the harvested cells were extracted using lysis buffer (9 M urea, 4% CHAPS, 40 mM Tris-HCl, 2% pharmylate, 40 mM DTT and 1 mM PMSF). The protein concentration in the supernatants was determined using the Non-Interference Protein Assay kit (Sangon Biotech, Shanghai, China) and then used for 2-DE. The total proteins (250  $\mu$ g) were loaded onto 24-cm non-linear IPG strips at pH 3-10 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) for simultaneous hydration, then isoelectric focusing (IEF) was performed using the Ettan™ IPGphor™ Isoelectric Focusing System (GE Healthcare Bio-Sciences) following the voltage-time program of 30 V for 13 h, 500 V for 1 h, 1,000 V for 1 h, 8,000 V for 3 h, 8,000 V for 44,000 Vh with a total power of 69,890 Vh. Following IEF, the strips were equilibrated for 2 intervals of 15 min in an equilibration buffer containing 6 M urea, 0.375 M Tris-HCl, 2% SDS, and 30% glycerol. The strips were then transferred onto the second-dimensional SDS-PAGE and run on 10% polyacrylamide gels at the program of 2.5 w/gel for 30 min; 15 w/gel for 5 h. The gels were fixed for silver nitrate staining prior to scanning.

**In-gel digestion and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS).** ImageMaster 2D Platinum analysis software (GE Healthcare Bio-Sciences) was used for spot detection, gel matching and spot quantification. Protein spots showing significant changes (protein spots with a ratio >1.5 or <0.5) following stimulation with TGF- $\beta$ 1 were manually excised and subjected to in-gel digestion. The digested samples were extracted and analyzed by MALDI-TOF/TOF MS (Sangon Biotech). For peptide mass fingerprinting (PMF), each mass spectrum was obtained from signals generated from at least 500 laser shots. The mass data were used to search the UniProt database (<http://www.pir.uniprot.org>) using the MS-Fit database search engine (<http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msfitstandard>).

**Bioinformatics analysis.** The miRNA sequences and annotation were accessible at miRBase 2.0 (<http://www.mirbase.org>). The potential miRNA targets for the selected miRNAs were screened using TargetScan 6.2 (<http://www.targetscan.org/>). The minimum free energy hybridization of the miRNAs and mRNAs was analyzed using the RNAhybrid tool (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid>). The secondary structure of single-stranded RNAs was predicted using the RNAfold WebServer (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>). The proteins identified by proteomics analysis were analyzed using the Mascot database (<http://www.matrixscience.com/>).

Table I. The differentially expressed miRNAs in GC-1 spg cells following stimulation with transforming growth factor-(TGF)- $\beta$ 1.

Differential expression	miRNA	Signal ratio (5 ng/ml vs. con)	Signal ratio (10 ng/ml vs. con)	Chromosome	Overlapping transcripts
Upregulation	mmu-miR-196a	3.8771	2.95	chr11 or chr15	Intergenic or Hoxc5 Mir196a
	mmu-miR-497	3.2642	2.7882	chr11	Intergenic
	mmu-miR-194	2.2598	-	chr1 or chr19	Iars2 or intergenic
	mmu-miR-199a-3p	2.2036	2.0962	chr1 or chr9	Dnm3os Dnm3 or Dnm2
	mmu-miR-199a-5p	2.0496	2.4190	-	-
	mmu-miR-181b	-	2.2239	chr1 or chr2	RP23 mmu-mir-181b or Nr6a1
	mmu-miR-149	-	2.2931	chr1	Gpc1
Downregulation	mmu-miR-3102-5p.2	0.4955	-	chr7	Arhgef17
	mmu-miR-320	0.4955	-	chr14	Intergenic
	mmu-miR-296-3p	0.4864	-	chr2	Intergenic
	mmu-miR-5105	0.4614	-	-	-
	mmu-miR-23a*	0.4604	0.4993	chr8	Intergenic
	mmu-miR-330*	0.4597	-	chr7	Eml2
	mmu-miR-351*	0.4572	-	chrX	Intergenic
	mmu-miR-503	0.4353	-	chrX	Intergenic
	mmu-miR-1941-5p	0.4295	0.3884	chr15	Krt80
	mmu-miR-877	0.4266	-	chr17	Abcf1
	mmu-miR-1943	0.3967	-	chr15	Tmem184b
	mmu-miR-5112	0.3604	0.3048	chr18	Intergenic
	mmu-miR-423-5p	0.3520	0.4899	chr11	Ccdc55
	mmu-miR-714	0.3312	-	-	-
	mmu-miR-700	0.2998	0.2706	chr4	Rcan3
	mmu-miR-3096-3p	-	0.3208	-	-
	mmu-miR-486*	-	0.3563	chr8	Ank1 AC126445.2 Gm15816

**Statistical analysis.** The data are presented as the means  $\pm$  SD (n=3). Statistical analysis was conducted using the Student's t-test. Differences were considered statistically significant at p-values  $\leq$ 0.05.

## Results

**Alterations in miRNA profiles induced by TGF- $\beta$ 1 in GC-1 spg cells.** Global miRNA expression levels induced by different doses of exogenous TGF- $\beta$ 1 in the mouse GC-1 spg cells, as shown by microarray analysis, are presented in Fig. 1. The differentially expressed miRNAs with a fluorescence signal of  $\geq$ 20 and fold changes of  $\geq$ 2 or  $\leq$ 0.5 (p $\leq$ 0.05) were analyzed using the t-test. Our data demonstrated that the expression of 24 out of approximately 924 miRNAs was altered following stimulation with TGF- $\beta$ 1. Of these, 7 miRNAs were upregulated and 17 miRNAs were downregulated (Table I). In addition, 4 miRNAs (mmu-miR-196a, miR-497, miR-199a-3p and miR-199a-5p) were upregulated following stimulation of the cells with 5 and 10 ng/ml TGF- $\beta$ 1, and 5 miRNAs (mmu-miR-23a\*, miR-1941-5p, miR-5112, miR-423-5p and miR-700) were downregulated following stimulation with the 2 different concentrations of TGF- $\beta$ 1. These miRNAs were mapped onto almost

all chromosomes and approximately 83% of these miRNAs were located in intronic or intergenic regions. In order to test the reliability of the microarray data, the expression patterns of 10 selected miRNAs, including 6 upregulated miRNAs and 4 downregulated miRNAs, were examined by RT-qPCR. The overall profile of miRNA expression by RT-qPCR analysis was similar to that revealed by the microarray data for all the selected miRNAs (Fig. 2).

**Alterations in protein profiles induced by TGF- $\beta$ 1 in GC-1 spg cells.** 2-DE and MALDI-TOF/TOF MS-based proteomics analysis were performed to explore the expression profiles of proteins associated with TGF- $\beta$ 1 signaling in mouse GC-1 spg cells. In the first dimension, the pH range of the IPG strips is from 3 to 10 with most of the proteins located between pH 4.0 and pH 8.0 (Fig. 3A). Along the second dimension, proteins are mainly distributed within the range of 20-70 kDa according to their molecular weight. In this study, protein spots with a ratio  $>$ 1.5 or  $<$ 0.5 were identified as differentially expressed. Of the identified proteins, 11 proteins were selected and subjected to MALDI-TOF/TOF MS of these differentially expressed spots. Fig. 3 shows the total number of identified proteins (Fig. 3A) and the downregulated protein spots (Fig. 3B). Table II shows

Table II. The characteristics of the downregulated proteins.

Spot No.	Protein/ gene name	Annotation	Proposed function <sup>a</sup>	MW/pI	Score	Sequence coverage	Protein alteration
2	TERA/ <i>Vcp</i>	Transitional endoplasmic reticulum ATPase	Involved in the formation of the tER, DNA damage, DNA repair, transport and Ubl conjugation pathway.	89950/5.14	622	17%	↓
4	GELS/ <i>Gsn</i>	Gelsolin	Plays a role in ciliogenesis.	86287/5.83	208	7%	↓
5	VINC/ <i>Vcl</i>	Vinculin	Involved in cell-matrix adhesion and cell-cell adhesion, and may also play an important role in cell morphology and locomotion.	117215/5.77	359	13%	↓
14	NDKB/ <i>Nme2</i>	Nucleoside diphosphate kinase B	Major role in the synthesis of nucleoside triphosphates other than ATP, negatively regulates Rho activity.	17466/6.97	409	47%	↓
15	COF1/ <i>Cfl1</i>	Cofilin-1/p18	Regulates actin cytoskeleton dynamics, plays a role in the regulation of cell morphology and cytoskeletal organization.	18776/8.22	464	46%	↓
16	PPIA/ <i>Ppia</i>	Peptidyl-prolyl cis-trans isomerase A	PPIases accelerate the folding of proteins.	18131/7.74	469	43%	↓
17	IF5A1/ <i>Eif5a</i>	Eukaryotic translation initiation factor 5A-1	mRNA-binding protein, involved in translation elongation and actin dynamics and cell cycle progression.	17049/5.08	674	58%	↓
18	EZRI/ <i>Ezr</i>	Ezrin	Probably involved in connections of major cytoskeletal structures to the plasma membrane. In epithelial cells, required for the formation of microvilli and membrane ruffles on the apical pole. Along with PLEKHG6, required for normal macropinocytosis.	69478/5.83	299	14%	↓

<sup>a</sup>The information regarding proposed function was obtained from UniProtKB (<http://www.uniprot.org/>). MW/pI, molecular weight/isoelectric point.

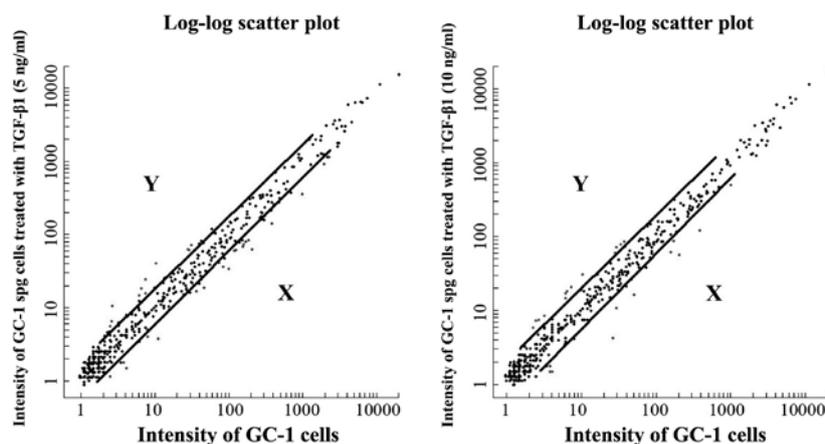


Figure 1. Alterations in microRNA (miRNA) expression profiles induced by transforming growth factor (TGF)- $\beta$ 1 in GC-1 spg cells. Scatter plots in region Y represent upregulated miRNAs, while scatter plots in region X represent downregulated miRNAs in GC-1 spg cells following stimulation with TGF- $\beta$ 1.

the relevant information of the downregulated polypeptides, including their annotation, function, molecular weight

(MW)/isoelectric point (pI), score and sequence coverage. The function of these identified proteins [gelsolin, vinculin,

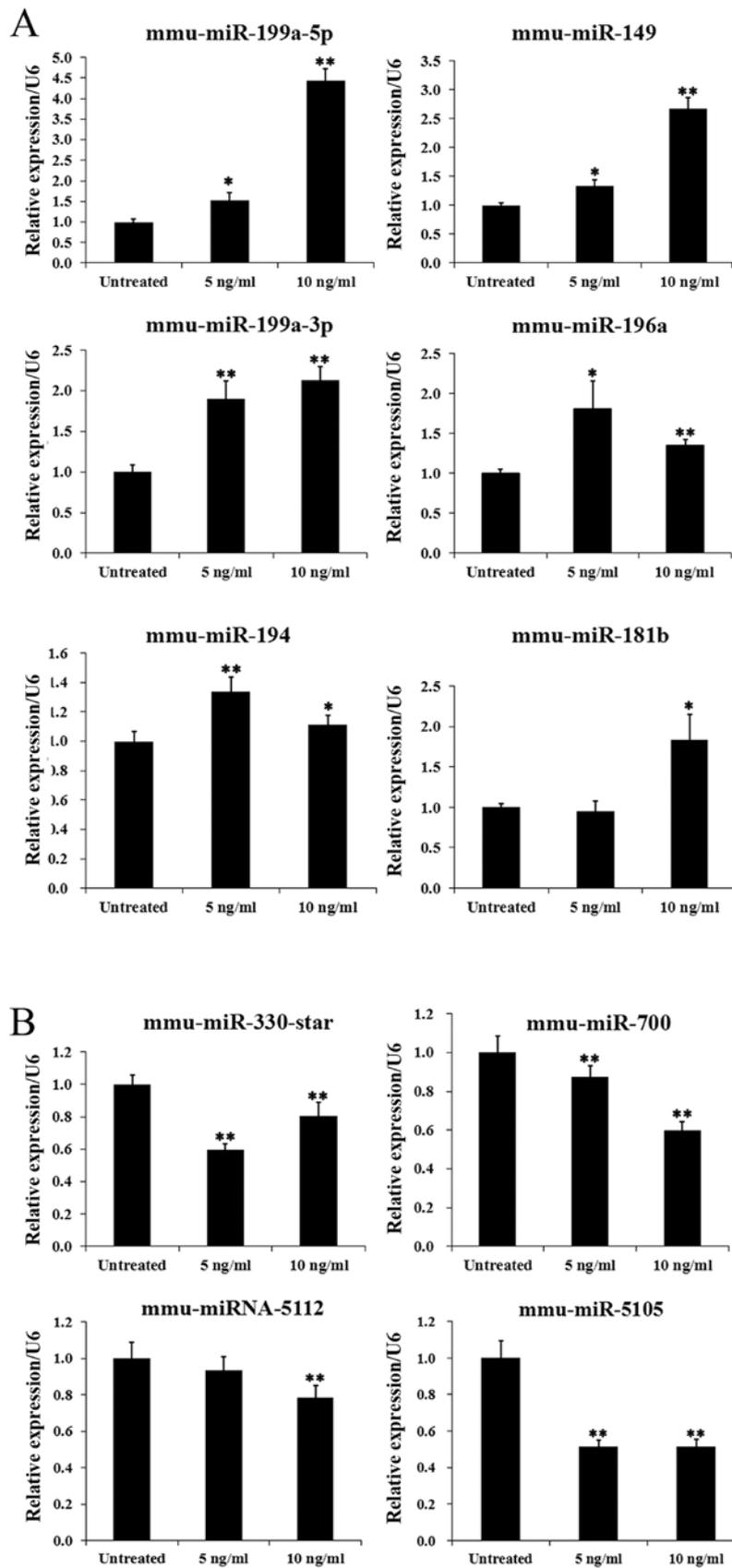


Figure 2. Verification of differentially expressed microRNAs (miRNAs) by RT-qPCR. (A) Upregulation, (B) downregulation (\*\* $P < 0.01$  and \* $P < 0.05$  vs. the control groups as shown by the t-test).

ezrin, nucleoside diphosphate kinase B (NDKB)/Nme2, peptidyl-prolyl isomerase A (PPIA), cofilin-1, transitional

endoplasmic reticulum ATPase (TERA) and eukaryotic translation initiation factor 5A-1 (IF5A1)] are mostly associated

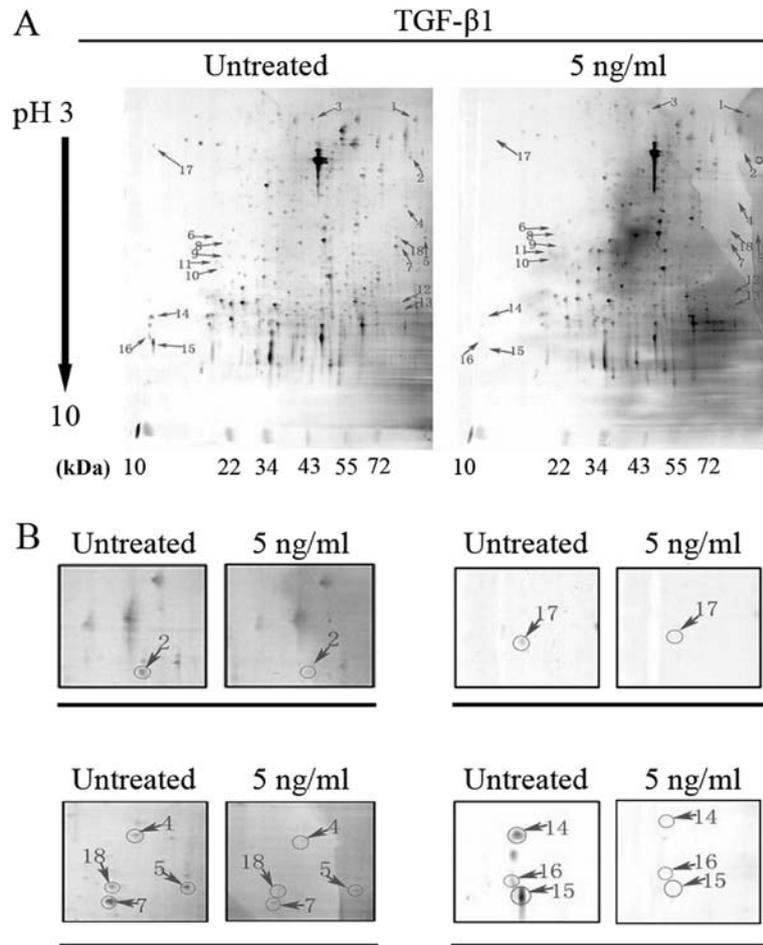


Figure 3. Alterations in protein expression profiles induced by transforming growth factor (TGF)- $\beta$ 1 in GC-1 spg cells. (A) Silver nitrate stained two-dimensional electrophoresis gels of untreated GC-1 spg cells and GC-1 spg cells with 5 ng/ml TGF- $\beta$ 1 induction. Molecular weights of markers were labeled on the bottom of gels. Arrows indicate altered protein spots. (B) Downregulation of 8 proteins was observed. Spot 2 was identified as TERA, spot 4 was identified as gelsolin, spot 5 was identified as vinculin, spot 14 was identified as NDKB/Nme2, spot 15 was identified as cofilin-1, spots 16 was identified as peptidyl-prolyl isomerase A (PPIA), spot 17 was identified as IF5A1 and spot 18 was identified as ezrin.

with invasion, cell-cell adhesion, actin dynamics, signal transduction and so on, suggesting that they may be involved in the cellular process of cell polarity, morphology and locomotion.

*Predicted miRNA binding sites of the selected proteins following stimulation with TGF- $\beta$ 1 in GC-1 spg cells.* Using miRBase 2.0 and TargetScan 6.2, the miRNA targets following the induction of TGF- $\beta$ 1 were predicted. It was predicted that miR-199a-3p would bind to target sequences in the 3'UTR of the mouse gene NDKB/Nme2 or Vcl mRNA, which was downregulated in the GC-1 spg cells following stimulation with TGF- $\beta$ 1 (Fig. 4A and C). Both miR-149 and miR-196a bind to target sequences in the 3'UTR of the downregulated gene, PPIA (Fig. 4E and G). These 3 miRNAs were upregulated in response to TGF- $\beta$ 1, as shown by miRNA microarray analysis. In addition, bioinformatics analysis further revealed that the minimum free energy hybridization of miR-199a-3p binding with the target gene NDKB/Nme2 (or Vcl) 3'UTR was markedly lower than that of the secondary structure of single-stranded NDKB/Nme2 (or Vcl) mRNA, indicating that NDKB/Nme2 and miR-199a-3p have a higher possibility for binding (Fig. 4B and D). We also analyzed this binding possibility between miR-149 and PPIA, or miR-196a and PPIA (Fig. 4F and H) and obtained similar

results. These results suggest that there may be a bio-functional link between these miRNAs and proteins.

## Discussion

The TGF- $\beta$ 1 superfamily comprises a broad range of signaling ligands, including TGF- $\beta$ 1, activin, nodal and bone morphogenetic protein (BMP), which regulate a variety of cellular processes, and its dysregulation often leads to cancer, male infertility and other diseases (4). Recently, miRNAs have emerged as major regulators of gene expression (14), and many more miRNAs have been identified and proven to be involved in various physical and pathological processes by regulating cell-fate decisions. In the present study, we performed miRNA microarray analysis in mouse GC-1 spg cells and identified a total of 24 miRNAs (Table I) that were regulated in response to stimulation of the cells with TGF- $\beta$ 1. Of these, 16 miRNAs are molecules associated with TGF- $\beta$ 1 signaling, while miR-181, miR-23a, miR-194, miR-199a and miR-196a family members have been previously reported to respond to TGF- $\beta$ 1 treatment in different cells and tissues (15-19). Taking the miR-199a family member as an example, researchers found that TGF- $\beta$ 1 increased the expression of miR-199a-3p in primary

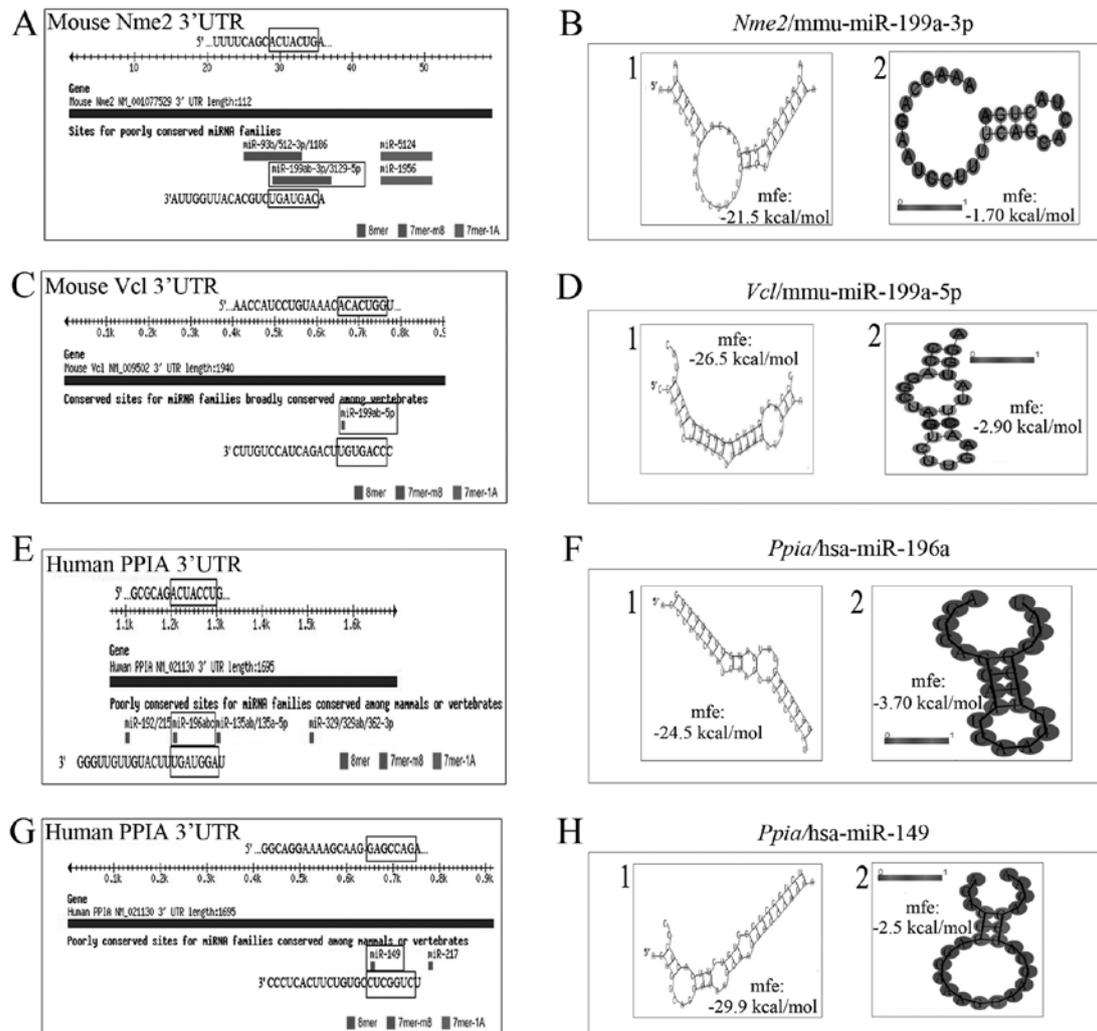


Figure 4. The selected proteins and their predicted microRNAs (miRNAs). (A, C, E and G) The predicted miRNA binding sites in 3'UTR of the target gene sequence. (B, D, F and H) The analysis of minimum free energy hybridization. Panel 1, the minimum free energy hybridisation of miRNAs and target gene mRNAs was predicted by RNAhybrid; panel 2, the secondary structure of single-stranded RNAs was predicted by the RNAfold WebServer.

human adult pulmonary fibroblasts and was associated with fibrotic remodeling (20). Furthermore, recent data suggest that miR-199a suppresses cell growth, cancer migration, invasion and metastasis in testicular cancer by regulating TGF- $\beta$ 1 signaling through the regulation of Smad4 (13). As the activation of TGF- $\beta$  signaling plays a central role in the pathogenesis of development, proliferation, differentiation, or apoptosis, the identification of these miRNAs regulated by TGF- $\beta$ 1 in GC-1 spg cells provides a possible link and a rationale for the hypothesis that they are downstream mediators of this pathway and mediate the functions of the pathway.

On the other hand, in order to identify candidate downstream target proteins in the GC-1 spg cells following stimulation with TGF- $\beta$ 1, we utilized comparative proteomics analysis to identify a group of 8 proteins, including gelsolin, vinculin, ezrin, NDKB/Nme2, PPIA, cofilin-1, TERA and IF5A1, which were suppressed following stimulation with TGF- $\beta$ 1 (Fig. 3). Some of these 8 proteins which were downregulated following stimulation with TGF- $\beta$ 1 have not been previously linked with TGF- $\beta$ 1 signaling. Bioinformatics analysis of these 8 candidate proteins using miRNA target prediction algorithms revealed that all 8 proteins contained more than one predicted miRNA binding

site. Of note, some predicted miRNAs, which targeted those 8 proteins were also identified by miRNA microarray analysis and their expression levels showed an opposite trend in response to stimulation with TGF- $\beta$ 1. More specifically, we noted that miR-149 and miR-199a-3p directly target PPIA and NDKB, respectively in response to stimulation with TGF- $\beta$ 1 in the mouse GC-1 spg cells, suggesting that these miRNAs play an important role in controlling downstream gene expression following the activation of TGF- $\beta$ 1 signaling. We therefore hypothesized that these proteins may be suppressed by the miRNAs in response to TGF- $\beta$ 1.

Nucleoside diphosphate (NDP) kinase B, also known as NME2, NM23B and NM23-H2, is an isoform of multifunctional proteins found to be responsible for the synthesis of nucleoside triphosphates in eukaryotes and is involved in a variety of cellular activities, including proliferation, development, adhesion and differentiation (21,22). NDKB is strongly expressed in the testis (23). High levels of NDKB have been observed at specific locations in post-meiotic germ cells, and its distribution is reminiscent of the microtubular structure of the manchette. In mature spermatozoa, NDKB is present at specific locations in the head and flagellar region. Normally,

during sperm differentiation, microtubules and motor proteins (cytosolic dynein and kinesin) of the manchette and the centrosome region require nucleotide synthesis for the spermatid nuclear shaping and sperm tail assembly (24,25). Studies have demonstrated an association of NDP kinases with microtubules and NDKB may thus have specific functions in the phosphotransfer network involved in spermiogenesis and flagellar movement (26,27). Our findings provide evidence of a direct mechanistic link of NDKB protein in TGF- $\beta$ 1 signaling, in which NDKB may be regulated by miR199a-3p, a member of an essential family that controls the fate of cell survival and death.

Another downregulated protein, PPIA, also known as cyclophilin A (CyPA), is an ubiquitously distributed protein belonging to the immunophilin family and regulates protein folding and trafficking (28). Although PPIA/CyPA was initially believed to function primarily as an intracellular protein, it has recently been shown that it can be secreted by cells in response to inflammatory stimuli (28). Another study demonstrated that PPIA/CyPA is present in rat germ cells, including pachytene spermatocytes, spermatids, interstitial cells and sertoli cell nuclei, and is associated with spermatocyte apoptosis (29). As TGF- $\beta$ 1 signaling plays a major role throughout development and in adult tissue homeostasis, our findings also provide evidence of a direct mechanistic link between PPIA protein and miR-149 or miR-196a in TGF- $\beta$ 1 signaling. This suggests the existence of some key mediators of the TGF- $\beta$ 1 pathway in early germ cell development.

In conclusion, we identified several important miRNAs and proteins as potential targets for TGF- $\beta$ 1 signaling. Of these miRNAs, miR-199a-3p and miR-149 (or miR-196a) were shown to directly target PPIA and NDKB and modulate the response to TGF- $\beta$ 1 signaling in the GC-1 spg cell line. The results obtained in this study may prove to be useful for further research on the function and mechanisms of action of miRNAs, as well as their targets which are involved in the process of TGF- $\beta$ 1-mediated spermatogenesis. Our data may lead to the development of novel therapeutic approaches for the treatment of male infertility.

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