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MicroRNA profiles and potential regulatory pattern during the early stage of spermatogenesis in mice

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Spermatogenesis is a complicated and poorly understood process that relies on the precise regulation of the self-renewal and differentiation of spermatogonia. In many organisms, microRNAs (miRNAs) are involved in multiple developmental processes as critical regulators of transcriptional and post-transcriptional gene silencing. This study investigated the expression pattern of miRNAs in type B spermatogonia cells (BSc) and primary spermatocytes (PSc) of mice, using a high-throughput small RNA sequencing system. The results revealed that the expression levels of Let-7 family miRNAs were remarkably high in both cell types. Furthermore, the expression levels of miR-21, miR-140-3p, miR-103, miR-30a, miR-101b and miR-99b were decreased during the transformation from BSc to PSc. These miRNAs target vital genes that participate in apoptosis, cell proliferation and differentiation, junction assembly and cell cycle regulation. These results highlight the indispensable role of miRNAs in spermatogenesis.

spermatogenesis, miRNA profiling, post-transcriptional regulation, focal adhesion, cytoskeleton dynamic, Wnt signaling pathway

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Spermatogenesis is a complex process in which spermatogonial stem cells form spermatozoa following mitotic, meiotic and post-meiotic phases. The process of spermatogenesis is highly sensitive to fluctuations in the environment and involves numerous endocrine and paracrine signals to coordinate the self-renewal of spermatogonial stem cells (SCCs) and spermatogonial differentiation [1]. Spermatogenesis is characterized by the phase-specific expression of many genes that are exclusively expressed in spermatogenic cells. With the development and application of technologies such as gene cloning, gene expression and functional characterization, many spermatogenesis-related genes have been identified in the past few years, some of which were found to play important roles in spermatogenesis [2]. Spermatogenesis-associated genes such as cyclins, proto-oncogenes and genes for azoospermia factor, cytoskeleton, heat shock proteins, nucleoprotein transition, centrin and apoptosis are involved in highly conserved landmark events such as meiotic recombination, formation of the synaptonemal complex, sister chromatid cohesion, spermiogenesis during post-

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meiotic stages, and checkpoints and factors required for the meiotic cell cycle.

Recently, small RNA molecules, including small interfering RNAs (siRNAs), microRNAs (miRNAs, approximately 22-nt sequences) and Piwi-interacting RNAs (piRNAs, 24- to 30-nt sequences) have emerged as important regulators of gene expression at the post-transcription or translation level [3]. Several miRNAs are expressed abundantly in male germ cells, either throughout or during specific stages of spermatogenesis [4]. piRNAs, which are actively involved in retrotransposon silencing that protects the integrity of the genome, are only present in pachytene spermatocytes and round spermatids [5]. piRNAs in type A spermatogonia, pachytene spermatocytes and round spermatids were profiled by deep sequencing in a recent study, the results showed that piRNA mapping to retrotransposons, mRNAs and intergenic regions had different length distributions and were differentially regulated in spermatogenesis [6]. In a recent study, mature mouse sperm were found to be extremely enriched in a novel class of tRNA-derived small RNAs (29–34 nt), which were slightly different from the RNA found in adult testes (26–32 nt). The discovery of sperm-borne RNAs have opened the possibility of additional paternal contributions aside from providing DNA [7].

miRNAs are a family of 21–25-nt cellular non-coding RNAs that bind to the 3′-untranslated region (UTR) of cognate mRNA through an imperfect match to repress their translation and stability [8, 9]. This is achieved by forming a ribonucleoprotein complex termed the RNA-induced silencing complex (RISC), which contains a member of the Argonaute family [10]. Recently, the fields of research on stem cells and miRNA have converged with the identification of stem cell-specific miRNAs [11,12]. Based on their function in translation attenuation, miRNAs seem to regulate the fate and behavior of stem cells by fine-tuning the protein levels of various factors required for stem cell or niche cell functions. Several miRNAs exhibiting tissue specificity have been identified in embryonic stem cells (ESCs) and somatic stem cells (SSCs) but not in germ-line stem cells (GSCs) [13]. Notably, loss of the RISC component Dicer in germ cells or Sertoli cells has been shown to interrupt germ cell development and lead to infertility [14]. This finding highlights the importance of miRNAs in the regulation of spermatogenesis. These studies provide information about a new layer of molecules associated with the intricate mechanisms of gene regulation, including miRNAs and RISC components, suggesting that miRNAs are functionally important in spermatogenesis.

In this study, we investigated the expression pattern and functional requirements of the miRNAs in type B spermatogonia and primary spermatocytes in order to illustrate their role in the early spatiotemporal development of spermatogenesis.

1 Materials and methods

1.1 Experimental cell lines

GC-1spg and GC-2spd (ts) cells [15,16] (ATCC, USA) were maintained in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1.5 g L^{-1} sodium bicarbonate. GC-1spg cells were created by transforming 10-day-old mouse type B spermatogonia with pSV3-neo; these cells exhibited characteristics of the stage between type B spermatogonia and primary spermatocytes. GC-2spd (ts) cells were created by transforming 6-week-old mouse spermatocytes with SV40 large T antigen; these cells lost their differentiation potential and remained arrested at the pre-meiotic stage. For simplicity, GC-1spg and GC-2spd (ts) cells were abbreviated as BSc and PSc respectively in the following results and discussion sections.

1.2 RNA extraction, library construction and sequencing

Total RNA was extracted from GC-1spg and GC-2spd (ts) cell lines by Trizol (Invitrogen, USA) according to the manufacturer's instructions. The RNA samples were incubated with 10 U DNase I (TaKaRa, China) for 30 min at 37°C to remove residual genomic DNA. The quality and quantity of the purified RNA were determined by measuring the absorbance at 260/280 nm (A_{260}/A_{280}) using a Nanodrop®ND-1000 spectrophotometer (LabTech, USA). The samples had an average RNA integrity number (RIN) value of 8.9, as determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Small RNAs (18–30 nt) were gel-purified from 10 μg total RNA. Adaptors were ligated to eluted RNAs successively with RNA ligase. Libraries were gel-purified after each ligation step. Small RNAs ligated with adaptors were subjected to RT-PCR (15 cycles of amplification) to produce sequencing libraries. PCR products were purified on a non-denaturing acrylamide gel and sequenced for 50 cycles on Illumina HiSeq 2000 Sequencer (BGI, China).

1.3 Analysis of sequencing data

Each tunnel generates millions of raw reads with a sequencing length of 49 bp. Sequencing data were mainly analyzed using the Short Oligonucleotide Alignment Program (SOAP) [17]. After eliminating low-quality reads, accurately clipping the adaptor sequences and removing reads whose lengths were smaller than 18 nt with the aid of a dynamic Perl programming algorithm, clean reads were mapped to mouse genome mm9. Reads were then screened against the Rfam and NCBI GenBank databases and classified into different types such as small RNAs, mRNAs and other types (i.e., rRNA, tRNA, scRNA, snRNA, snoRNA, repeat-associated small RNAs and so on). Known miRNAs

such as small RNAs that perfectly matched the miRBase (version 18.0) mouse miRNA precursor sequences and those identified as known miRNA editing polymorphisms were annotated and calculated to only one category according to priority.

1.4 Detection of differentially expressed miRNAs

We compared the expression profiles of the identified miRNAs in both samples. The expression level was normalized using TPM for calculating the fold change between GC-1spg and GC-2spd (ts) cell lines. Differentially expressed miRNAs were identified using a rigorous algorithm as described previously [18]. Fold change between the two samples were calculated, if the log2 value of the fold change between two samples was greater than 1 or less than −1, and the *P*-value was less than 0.05, the miRNA was considered to be a potentially differentially expressed miRNA.

1.5 Prediction of miRNA target genes and enrichment analysis of mouse KEGG pathways

The predicted target genes of all mouse miRNAs were downloaded from the miRWalk database, the prediction results combine comparative analysis of five prediction programs—DIANA-mT, miRanda, miRDB, RNA22 and TargetScan [19] and parameters for searching the miRWalk were as follows: Gene region 3′-UTR, min seed length 7, *P*-Value 0.05, Transcript longest. Our own Perl script was used to filter the combination results. Enrichment analysis of KEGG pathway revealed the main pathways in which the candidate target genes are involved. This analysis identifies significantly enriched metabolic pathways or signal transduction pathways in which the candidates of target genes are involved by comparing the genes with the whole reference gene background. Two-sided Fisher's exact test corrected by multiple testing and the χ^2 test were used to classify the pathway category. FDR was used to correct the *P*-value, and only pathway categories with corrected *P*-value ⩽0.05 were selected as enriched ones.

1.6 Stem-loop RT-PCR

Total RNA was extracted from GC-1spg and GC-2spd using the same methods as mentioned in RNA extraction and library construction. For the RT-PCR, the reaction mixture contained purified total RNA, cell lysate or heat-treated cells as the RNA samples, 50 nmol L^{-1} stem-loop RT primer, 1×RT buffer (Epicentre, USA), 0.25 mmol L^{-1} of each dNTP (HyTest Ltd, Finland), 2 U μL^{-1} MMLV reverse transcriptase (Epicentre, USA) and 0.25 U μL^{-1} RNase inhibitor (Epicentre, USA). The 7.5-μL reaction mixtures were incubated in an GeneAmp PCR System 9700 (Applied Biosystems, USA) in a 96- or 384-well plate for 30 min at 16°C, 40 min at 42°C, 5 min at 85°C and then held at 4°C. All reverse transcriptase reactions, including no-template controls and RT minus controls, were run in duplicate.

Real-time PCR was performed using a standard TaqMan® PCR kit protocol on an Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems). The 10-μL PCR mixture consisted of 0.67 μL of RT product, 1×TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.2 μmol L^{-1} TaqMan® probe, 1.5 μmol L^{-1} forward primer and 0.7 μmol L^{-1} reverse primer. The reactions were incubated in a 384-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were run in triplicate. The threshold cycle (C_t) was defined as the fractional cycle number at which fluorescence passes the fixed threshold. The average C_t value was calculated for each sample using the $2^{-\Delta\Delta C_t}$ method, normalized to U6, and expressed relative to spermatogonia [20].

2 Results

2.1 Overview of small RNA sequencing results

After preparation of small RNA sequencing libraries, Illumina high-throughput sequencing was used to characterize all small RNA species in both BSc and PSc. Initially, we obtained 17209025 and 15352366 raw reads for BSc and PSc respectively. After excluding reads containing ambiguous bases, adaptor contaminants and short reads, 13512566 (79.17%) and 9283464 (60.95%) clean reads were left for BSc and PSc respectively. Clean reads from both BSc and PSc showed exclusive length distribution with a distinct peak at 19–22 nt (Figure 1A and B). In total, 9914986 (73.38%, BSc) and 6633665 (71.46%, PSc) clean reads could be mapped to the mouse mm9 genome. In BSc, 6285296 (46.51%) clean reads were identified as miRNAs, while this number was 3126965 (33.68%) in PSc. Reads that could not be classified to any known RNA groups formed a considerable proportion of the annotation results at 2116886 BSc (15.67%) and 1126311 PSc (12.13%; Figure 2A, B and Table S1 in Suppporting Information).

2.2 Abundant expression of mmu-let-7 family in both BSc and PSc

The expression value of each known miRNA was normalized by TPMs (transcripts per million). Of 717 miRNAs checked, 322 miRNAs were expressed in at least one of the two cell lines. The results revealed that in BSc, 42.2% (136) of the miRNAs were poorly expressed (with TPMs<10), 54.7% (176) miRNAs were moderately expressed (with TPMs 10–10000), and only 3.1% (10) miRNAs were abundantly expressed (with TPM >10000). These 10 miRNAs accounted for 75.6% (4925373) of all miRNA reads. In PSc, the number of poorly, moderately and abundantly expressed miRNAs are 163 (50.6%), 154 (47.8%) and 5 (1.6%) re-

spectively, the highly expressed miRNA reads accounting for 65.9% (2181566) of all miRNA reads (Table S2).

Interestingly, reads mapping to mmu-let-7a, mmu-let-7b, mmu-let-7c, mmu-let-7d, mmu-let-7e, mmu-let-7f, mmu-let-7g and mmu-let-7i accounted for 57.2% (3952243) and 66.8% (2320407) of all miRNA reads in BSc and PSc, respectively (Table S2), which means that most members in mmu-let7 family play fundamental roles in maintaining status of these two cell lines. Among the eight mmu-let-7 family members detected, mmu-let-7g and mmu-let-7i were significantly down-regulated in PSc, indicating they may exert regulatory effect during the transformation from BSc to PSc (Table 1). Let-7 family miRNAs are thought to be master regulators of cell proliferation pathways [21], our findings in BSc and PSc show that let-7 family may play indispensable role in GSC development.

2.3 Differentially expressed miRNAs distinct from other types of stem cells

Totally, 179 miRNAs were detected as differentially expressed, of which 142 miRNAs were down regulated and 37 were up regulated in PSc (Figure 3, Table S2). We intended to determine which miRNAs, especially those that have not yet been reported, play a major role in GSC (germ stem cell) development. Forty miRNAs with TPM>100 in either

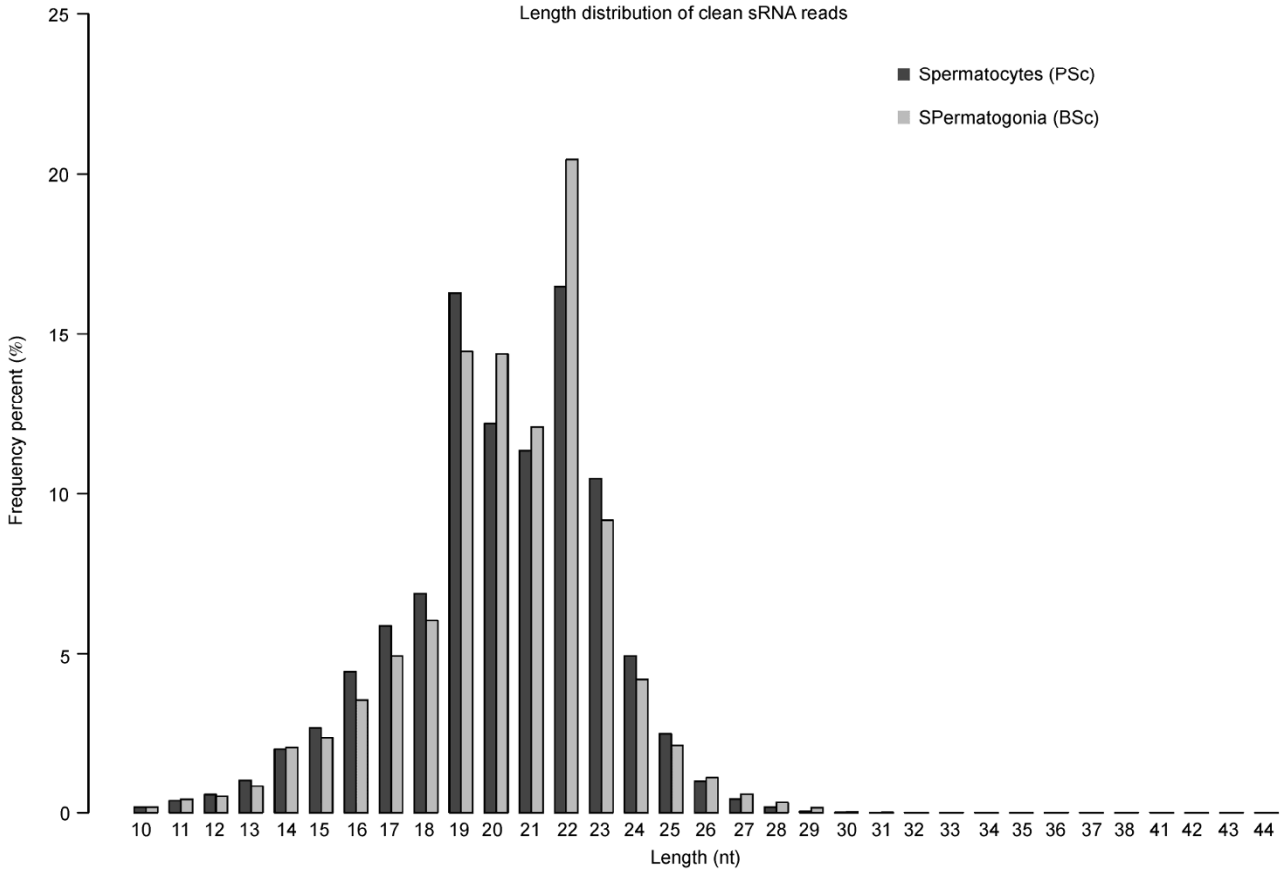

Figure 1 Length distribution of all clean small RNA reads of type B spermatogonia cells (BSc) and primary spermatocytes (PSc).

Table 1 Let-7 family expression among BSc and PSc

miR-name	PSc (TPM)	BSc (TPM)	Fold-change (log2 BSc/PSc)	*P*-value	Sig-label
mmu-let-7a	44323.7567	43445.3382	–0.02887878	7.58×10^{-23}	
mmu-let-7b	48413.7171	38769.8384	–0.32048115	0	
mmu-let-7c	102487.2828	124275.4337	0.27809623	0	
mmu-let-7d	8888.6002	9752.3298	0.13379068	1.51×10^{-97}	
mmu-let-7e	19016.5007	22532.3599	0.24474663	0	
mmu-let-7f	20753.5678	36147.3905	0.80053213	0	
mmu-let-7g	2927.3556	7043.7399	1.26674363	0	**
mmu-let-7i	3139.7763	10520.0596	1.7444092	0	**

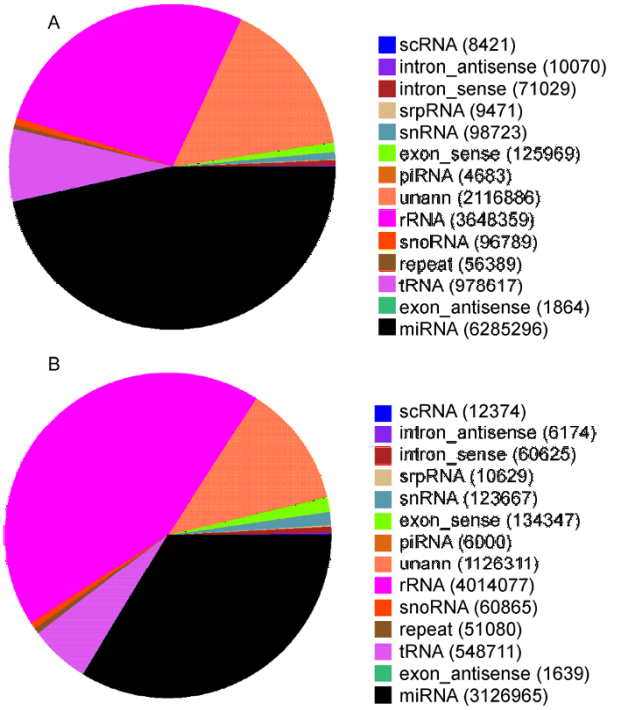

Figure 2 Annotation of RNA classification on all clean small RNA reads in both type B spermatogonia cells (BSc) (A) and primary spermatocytes (PSc) (B).

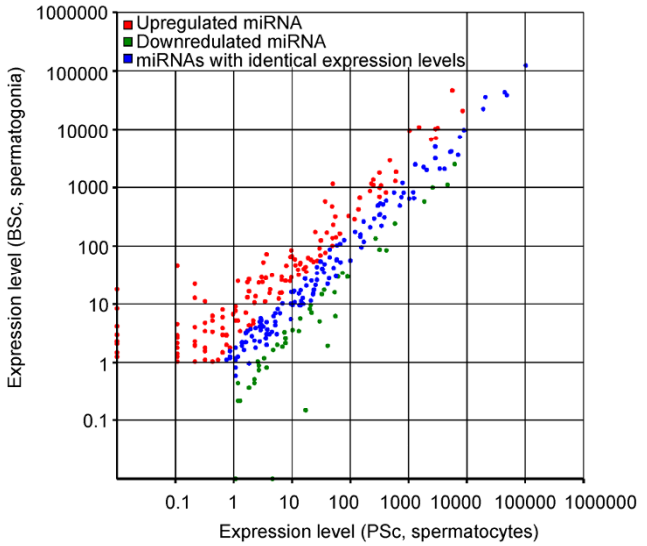

Figure 3 Scatter plot of all detected miRNAs' expression levels of type B spermatogonia cells (BSc) and primary spermatocytes (PSc).

BSc or PSc were termed as significantly differentially expressed miRNA (SDE miRNA). Among these 40 SDE miRNAs, 10 were with TPM>2000 in either of the cell lines, they were considered to play important roles during early GSC development (Table S2). Among them, mmu-let-7i, mmu-miR-140-3p, mmu-miR-21, mmu-miR-103, mmu-miR-30a and mmu-miR-101b were expressed with TPM of almost near or greater than 10000 in BSc but were remarkedly lower in PSc than in BSc.

Currently, no miRNAs dedicated specially to GSCs have been discovered. The miR-290-295, miR-17-92 and miR-15b-16 clusters have been reported to be vital for self-renewal of ESCs in mice, while miR-22 was found to be vital for differentiation of ESCs [12,22]. Of these three clusters, miR-290-295 cluster was not expressed in either BSc or PSc in our study (Table S2). miRNAs in the miR-17-92 cluster were down-regulated during the transition from BSc to PSc, albeit to different extents. Except for mmu-miR-92a, members of the miR-17-92 cluster were all expressed with TPMs<1000 (Table S2). Among miRNAs of the miR-15b-16 cluster, miR-15b was up-regulated by nearly two-fold in PSc, but miR-16 showed no apparent difference. MiR-22 also showed no difference between BSc and PSc (Table S2).

2.4 Functional enrichment analysis of target genes of SDE miRNAs and candidate marker genes regulated by SDE miRNAs

We focused on the target genes of SDE miRNAs and explored their potential roles during germ stem cell development. Because different genes usually cooperate with each other to play different roles in biological functions, pathway-based analysis could aid in obtaining a better understanding of the biological function of genes. Based on the target prediction results of all SDE miRNA on the miRWalk website, we checked the combined prediction results of five programs—DIANA-mT, miRanda, miRDB, RNA22 and TargetScan. We call it a miRNA-target pair if the targeting relationship is supported by at least two programs. Using this strict filtering conditions, 40 SDE miRNA were predicted to target 12835 genes (Table S3). All these target genes were checked using our published DGE (digital gene expression) data generated from the same cell lines [23] to filter out genes which were not differentially expressed, or which showed the same expression pattern as corresponding miRNAs. 1437 genes passed the filtering, which means that these DEGs are highly prone to be regulated by corresponding miRNAs and they were termed miRNA-targeted-DEGs (Table S3).

Through functional enrichment analysis of KEGG pathways, we found that the 1437miRNA-targeted-DEGs were enriched in four pathways they were pathways in cancer, apoptosis, gap junction and focal adhesion respectively (corrected P<0.05; Table 2). Among these four pathways, the DEGs detected between BSc and PSc were found to be also enriched in three of them—pathways in cancer, apoptosis and focal adhesion, which indicates that these functionally important pathways during the transition from BSc to PSc are potentially affected or regulated by SDE miRNA.

Among the target genes in the four enriched pathways, FGF7, FGF13 and WNT10A in mmu05200—pathways in cancer, were reported as candidate markers which are dif-

ferentially expressed on both mRNA and protein levels and can discriminate BSc from PSc [23]. Based on the target prediction results, FGF7 was up-regulated in PSc and was targeted by mmu-miR-503 and mmu-miR-340-5p. FGF13 and WNT10A were both down-regulated in PSc, FGF13 was targeted by mmu-miR-143 and WNT10A was targeted by mmu-miR-206 and mmu-miR-143 (Table 3).

2.5 Experimental validation of potential miRNA markers

As shown above, of the 40 significantly differentially expressed miRNAs identified in this study, mmu-let-7i, mmu-miR-140-3p, mmu-miR-21, mmu-miR-103, mmu-miR-101b and mmu-miR-30a were expressed with TPM values of almost or greater than 10000 in BSc but with extremely low TPM in PSc. Stem-loop reverse transcription (RT)-polymerase chain reaction (PCR) analysis was used to verify the expression levels of these six miRNAs (Table S4). The RT-PCR results of the six miRNAs correlated well with the sequencing results (Figure S1), indicating that these six miRNAs were truly dramatically changed and they may be used as candidate miRNA markers to distinguish BSc from PSc.

3 Discussion

3.1 Germ cell lines as models to study the early stage of spermatogenesis

Study of gametogenesis and meiotic process that is fundamental for reproduction and the maintenance of genetic diversity require establishing mammalian germ-cell lines capable of differentiation *in vitro*. Hofmann et al. [16] had reported the immortalization of all the cell types contributing to a developing seminiferous tubule in mouse testis using the SV40 large T antigen. These cell types include 16 peritubular, 22 Leydig, 8 Sertoli, and 1 germ cell line, the germ cell line (GC-1spg) was established that corresponds to a stage between spermatogonia type B and primary spermatocyte, based on its characteristics in phase contrast and electron microscopy. These four immortalized cell types are able to reaggregate and form structures resembling two-dimentional spermatogenic tubules *in vitro*. This means that these immortalized cells can exert similar functions as they are *in vivo*. GC-2spd(ts) were obtained by mouse primary germ cells enriched in preleptotene spermatocytes cotransfected with the plasmid pSV3neo containing the LTAg gene and the plasmid LTRp53cG9 containing the temperature sensitive p53 gene ((ts)p53) [15]. At permissive temperature of 37 degrees, the GC-2spd (ts) cell line generates cells with a haploid DNA content and morphologic and biochemical features of round spermatids. Since both GC-1spg and GC-2spd (ts) are able to proliferate over long periods of time and to differentiate *in vitro*, they would greatly facilitate the identification and characterization of the factors that induce germ-cell proliferation or meiosis. It is appropriate that we used these two cell lines to test the roles played by miRNA during early stage of mouse spermatogenesis.

Table 2 Enriched pathways of the target genes of SDE miRNAs

Term	*P*-Value	Genes	Bonferroni correction
mmu05200: Pathways in cancer	7.87×10^{-6}	*E2F1*, *FGF5*, *FGF7*, *PDGFB*, *PDGFA*, *FGF11*, *FGF13*, *GLI3*, *MMP2*, *CCNE2*, *FOS*, *RALA*, *FAS*, *WNT6*, *AKT2*, *WNT10A*, *WNT10B*, *RUNX1T1*, *CDK6*, *PIAS2*, *WNT11*, *LAMC2*, *LAMC1*, *WNT9A*, *XIAP*, *PML*, *EGLN3*, *NFKBIA*, *EGLN2*, *PIK3R5*, *AXIN2*, *RUNX1*, *TRAF6*, *FIGF*, *FN1*, *APC*, *EPAS1*, *BRCA2*, *IGF1*, *SMAD2*, *BIRC2*, *KITL*, *FZD7*, *DVL1*, *NRAS*, *ITGA6*, *RASSF1*, *JAK1*, *WNT7A*	0.0014
mmu04210: Apoptosis	1.04×10^{-4}	*IRAK1*, *XIAP*, *NFKBIA*, *ENDOD1*, *CAPN2*, *BIRC2*, *PRKAR2B*, *TNFRSF1A*, *IRAK3*, *MYD88*, *TNFRSF10B*, *PRKAR1B*, *PPP3CB*, *PIK3R5*, *PRKACA*, *PRKACB*, *FAS*, *NGF*, *AKT2*	0.0180
mmu04540: Gap junction	2.88×10^{-4}	*GNAI3*, *GNAI2*, *PDGFB*, *ADCY7*, *GNAI1*, *PDGFA*, *GNA11*, *PRKG2*, *LPAR1*, *GRM1*, *NRAS*, *ADCY9*, *TUBA4A*, *PRKACA*, *GNAS*, *PDGFD*, *PRKACB*, *TUBB3*	0.0491
mmu04510: Focal adhesion	2.98×10^{-4}	*2900073G15RIK*, *XIAP*, *PDGFB*, *PDGFA*, *PIP5K1C*, *ELK1*, *ITGB3*, *DOCK1*, *COL6A1*, *PIK3R5*, *ZYX*, *PAK1*, *PDGFD*, *THBS1*, *FIGF*, *COL11A1, AKT2, FN1, FLT1, ROCK2, IGF1, CAPN2, BIRC2, COL5A2, ITGA6, RAP1A, RAP1B, LAMC2, LAMC1, PARVB, MYLK*	0.0508

Table 3 Candidate marker genes which can discriminate type B spermatogonia cells (BSc) and primary spermatocytes (PSc) and their potential corresponding miRNA regulators

DEG marker	DEG pattern	miRNA	miRNA pattern
Fgf7	↑	mmu-miR-503, mmu-miR-340-5p	↓
Fgf13	↓	mmu-miR-143	↑
Wnt10a	↓	mmu-miR-206, mmu-miR-143	↑

3.2 Highly expressed mmu-let-7 family in BSc and PSc implies their involvement in early stage of spermatogenesis

In this study, we found that seven members of the mmu-let-7 miRNA family were the most abundantly expressed miRNAs in both BSc and PSc; in particular, mmu-let-7a–f and let-7i were the most abundantly expressed miRNAs. mmu-let-7a–f accounted for a large proportion of all the miRNAs (80% in the case of BSc and 82.3% in PSc), mmu-let-7g and mmu-let-7i showed significantly differential expression pattern between BSc and PSc. As one of the first two known miRNAs (the other one is lin-4) [24,25] conserved in invertebrates and vertebrates, let-7 has been extensively studied. It controls the timing of cell cycle exit and terminal differentiation in *Caenorhabditis elegans* and is poorly expressed or deleted in human cancers and cancer stem cells, acting as a tumor suppressor [26]. As one of the four genes involved in induced pluripotent stem (iPS) cell reprogramming [27], LIN28 expression is the opposite to that of mature let-7 [28]. Let-7 might enhance its own level by repressing its negative regulator LIN28 [29–31]. Microarray analysis has revealed many genes regulating cell cycle and cell proliferation that are responsive to alterations in let-7 expression levels, including cyclin A2, CDC34, Aurora A and B kinases (STK6 and STK12), E2F5 and CDK8 [32]. Subsequent experiments have confirmed the direct effects of some of these genes, such as CDC25A and CDK6 [26]. Let-7 also inhibited several components of the DNA replication mechanism, transcription factors and even some tumor suppressor genes and checkpoint regulators [32]. Indeed, let-7 family members are also abundant in the testes [33] and spermatozoa [34], suggesting that let-7 family members may contribute to the regulation of the male germ cell lineage. The extreme activity of most let-7 family members in both BSc and PSc demonstrates their importance to early stage of spermatogenesis. Additionally, our findings demonstrate that mmu-let-7g and mmu-let-7i may be particularly crucial during differentiation from BSc to PSc, as only their expression level dropped sharply across these two cell lines.

3.3 Potential marker miRNAs may distinguish BSc from PSc

The expression levels of mmu-miR-21, mmu-miR-140-3p, mmu-miR-103, mmu-miR-30a and mmu-miR-101b were close to those of the let-7 family members and showed significant decreases during the transition from BSc to PSc, indicating their active involvement in the transition from BSc to PSc. The significant expressional difference between the two cell lines suggests that these miRNAs may be key regulators of the transition from BSc to PSc. These miRNAs were down-regulated to regulate the expression of corresponding target genes which promote the transformation from mitotic to meiotic state. miR-21 was one of the first mammalian miRNAs to be identified, and it is active in many processes such as apoptosis [35], development [36], oncology [37] and stem cells [14]. Furthermore, miR-140 has been shown to be essential for normal endochondral bone development and proliferation [38], while miR-103 is linked to insulin sensitivity [39] and intestinal cell proliferation [40]. Recently, miR-30a has been found to suppress tumor growth in colon carcinoma by targeting DTL gene [41]. MiR-101b cooperates with let-7 miRNAs in tumors to inhibit cell proliferation [42], and it also participates in the FGF-2-regulated cell proliferation, migration and angiogenesis through an NDY1/KDM2B-miR-101-EZH2 pathway [43]. Among the six candidate miRNA markers, miR-21 has been found to be involved in the differentiation of ESCs [22] and to target the Nanog and Sox2 genes [44]. One report has shown that miR-103 cooperated with miR-107 in hematopoiesis from SSCs [45]; however, no studies have described the involvement of any of the other three miRNAs in stem cell development.

These potential marker miRNAs are related to developmental processes, including proliferation and differentiation. As stem cell specific miRNAs showed distinct profiles in specific cell types or during specific stages of differentiation [13], miRNAs that were differentially expressed in BSc and PSc but distinct from the other known stem cell-related miRNAs can be regarded as the candidate marker miRNAs that may distinguish spermatogonia from spermatocytes. Further studies are required to elucidate the specific roles of these miRNAs in the early stage of spermatogenesis.

3.4 Interconnected relationship between differentially expressed miRNAs and their target genes involved in the transition from BSc to PSc

miRNAs perform their function by targeting corresponding mRNAs coding for important proteins in vital pathways of the developmental process, and regulating their expression levels spatiotemporally. Many of the genes and transcription factors potentially involved in spermatogenesis have already been identified and most are essential for regulating the early mitotic phase of spermatogenesis [46]. In this study, we compared the miRNA expression between BSc and PSc, the target genes of significantly differentially expressed miRNAs should be closely related with the regulation of spermatogenesis. Many known targets of these miRNAs and their functions have been studied. The known target genes barely participate explicitly in spermatogenesis-related pathways, and the knowledge of these genes is restricted due to the poor understanding of the relationship between miRNAs and the development of GSCs.

Using DGE (digital gene expression tag profiling) analysis on the same cell lines used in this study, Zhang et al. [23] have found that pathways related to focal adhesion, pathways in cancer, axon guidance, apoptosis and so on are rel-

evant to early stage of spermatogenesis. These pathways may cooperate with each other to regulate and promote the differentiation from BSc to PSc. Also, some candidate marker genes which are differentially expressed were experimentally tested further on protein expression level. In this study, miRNA and target prediction results indicated that FGF7, FGF13 and WNT10A may be regulated by some SDE miRNAs, further functional study is needed to test this conjecture. Pathway enrichment analysis of SDE miRNAs' targets indicated that miRNAs may play important post-transcriptional regulatory roles during the transformation from BSc to PSc, especially on pathway in cancer, apoptosis, gap junction and focal adhesion signaling pathways. Except for gap junction pathway, the other three pathways are all significantly affected during the transition from BSc to PSc, which means that these SDE miRNAs may cooperate with each other to involve in the regulation network. Real relationship between these SDE miRNA or candidate marker miRNA and the functional pathways needs further investigation.

The authors declare that they have no conflict of interest.

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Supporting Information

Figure S1 Significantly differentially expressed miRNAs between of type B spermatogonia cells (BSc) and primary spermatocytes (PSc) validated by stem-loop RT-PCR experiments.

Table S1 Statistics of deep-sequencing data of small RNA libraries of BSc and PSc.

Table S2 Expression levels of all detected miRNAs and miRNAs expressed at extremely high levels in either BSc or PSc.

Table S3 All differentially expressed miRNA and their corresponding target genes.

Table S4 Primers for stem-loop real-time PCR of 6 marker miRNAs.

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