Microarray profiling of microRNAs expressed in testis tissues of developing primates

Naihong Yan · Yilu Lu · Huaqin Sun · Weimin Qiu ·
Dachang Tao · Yunqiang Liu · Huijiao Chen ·
Yuan Yang · Sizhong Zhang · Xiang Li · Yongxin Ma

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Abstract

Purpose MicroRNAs (miRNAs) are small non-coding RNA molecules that have been identified as potent regulators of gene expression. Recent studies indicate that miRNAs are involved in mammalian spermatogenesis but the mechanism of regulation is largely unknown.

Methods miRNA microarray was employed to compare miRNA expression profiles of testis tissues from immature rhesus monkey (Sample IR), mature rhesus monkey (Sample MR), and mature human (Sample MH). Real-time RT-PCR was used to confirm the changed miRNAs.

Results Twenty-six miRNAs were shared by samples IR/MR and IR/MH with differential expression patterns greater than three-fold difference. PicTar and TargetScan prediction tools predicted a number of target mRNAs, and some of these target genes predicted by miRNAs have been shown to associate with spermatogenesis.

Conclusions Our results indicate that miRNAs are extensively involved in spermatogenesis and provide additional information for further studies of spermatogenetic mechanisms.

Keywords Spermatogenesis · qRT-PCR · PicTar and TargetScan · Gene expression

Introduction

It has been estimated that approximately 15% of couples worldwide suffer from infertility and about half of these cases are due to defective spermatogenesis [1]. Spermatogenesis is the development and differentiation of germ cells that take place in the seminiferous tubules of the mammalian testis. In spermatogonial germ cells, a temporal disconnection between transcription and translation is especially common [2]. Thus, post-transcriptional mechanisms play major roles in the temporal regulation of protein synthesis in developing male gametes. The major cellular changes are strongly dependent on post-transcriptional regulatory processes due to early termination of transcription in the haploid phase of germ cell maturation [3].

Recently, miRNA-mediated translational regulation has emerged as a distinct mechanism of post-transcriptional regulation. miRNAs are an abundant class of non-coding RNAs ranging from 20 to 23 nt in length and function as post-transcriptional regulators of gene expression by influencing translation and stability of mRNAs [4]. The 5'-seed region of a miRNA (the 5' end of miRNA with conserved 8mer and 7mer sites) interacts with the 3' untranslated region (3'-UTR) of a target mRNA by partial sequence complementarity, resulting in the degradation of the target mRNA and thus translational inhibition [5]. Expression of miRNAs appears to be highly regulated by developmental stage and tissue specificity, although little is known about the role of miRNAs in germ cells.

Evidence shows that miRNAs may constitute a major mechanism of translational regulation during spermatogenesis in mice. miRNAs are expressed in male germ cells and

Capsule This work, based on microRNA microarray assay and qRT-PCR assay, predicted genes associated with spermatogenesis by microRNA analysis.

N. Yan · Y. Lu · H. Sun · W. Qiu · D. Tao · Y. Liu · H. Chen ·
Y. Yang · S. Zhang · X. Li · Y. Ma (✉)
Department of Medical Genetics, West China Hospital,
Division of Human Morbid Genomics,
State Key Laboratory of Biotherapy, Sichuan University,
Chengdu, People's Republic of China
e-mail: mayongxing@263.net

N. Yan
Laboratory of Ophthalmology, West China Hospital,
Sichuan University, Chengdu, People's Republic of China

essential for the completion of spermatogenesis [6], but their regulatory functions during germ cell development remain uncertain. Many testicular miRNAs have been cloned by several groups [7–9]. Further studies identified some testicular miRNAs that are involved in the regulation of gene expression. One such miRNA, miR-122a, targets the 3'-UTR of the transition protein 2 (*Tnp2*) mRNA, a post-transcriptionally regulated testis-specific gene involved in chromatin remodeling during spermatogenesis of mice [6].

Spermatogenesis is heavily dependent on post-transcriptional regulatory processes, such as gene silencing via perfect or imperfect base-pairing of miRNAs to the 3'-UTR of target mRNAs [10]. However, the functions of miRNA pathways as well as the expression and localization of the components of these pathways during spermatogenesis remain unknown. The identification of the entire set of miRNAs and their target genes from testis tissues is physiologically and pathologically significant in order to understand the molecular mechanism of spermatogenesis. In this study, to determine miRNA expression levels during spermatogenesis, microarray technique was employed on testis tissues of immature rhesus monkey (IR), mature rhesus monkey (MR), and mature human (MH). We analyzed 1,172 miRNAs from the three samples and found significant changes in a large number of miRNAs, among which 26 were shared by samples IR/MR and IR/MH and further confirmed by qRT-PCR [11, 12].

miRNAs are involved in the regulation of protein expression primarily by binding to one or more target sites on target mRNA transcripts, resulting in the degradation of mRNA and inhibition of translation. Thus, the identification of target mRNAs is the most important aspect in understanding miRNA function. Computational algorithms have become a crucial tool in the prediction of miRNA-regulated transcripts, such as PicTar (<http://pictar.bio.nyu.edu/>) and TargetScan (<http://www.targetscan.org/>) [13, 14]. From the 26 miRNA shared by samples IR/MR and IR/MH, 15 were also shared by samples MR/MH and account for the difference between immature and mature testis miRNA expression. A large number of target genes for 14 of the 15 miRNAs (excluding PREDICTED_MIR165) were predicted by PicTar and TargetScan prediction tools. Some of the predicted target genes are associated with spermatogenesis as validated *in vitro* and *in vivo*.

Materials and methods

Rhesus monkey testis tissues

Juvenile (about 1 year old and weighing 1.95 kg) and adult (about 6 years old and weighing 7.62 kg) male rhesus monkeys

(*Macaca mulatta*) were obtained from PingAn Monkey Breeding Base of Chengdu through National Chengdu Center for Safety Evaluation of Drugs (NCCSED), accredited by Association for Assessment and Accreditation of Laboratory Animal Care international (AAALAC). The experiments described in this study were conducted according to the Guide for the Care and Use of Laboratory Animals (National Research Council, Chinese Version, 1996). Animals were reared in the animal care facilities of NCCSED under a 12-hour light-dark lighting cycle. Immature (IR) and mature (MR) testis tissues from juvenile and adult monkeys were collected and frozen in liquid nitrogen immediately following euthanizing the animals with sodium pentobarbital overdose.

Human testis tissues

Adult human testis tissues (MH) were recovered from a 30-year-old male registered in our local organ donation program. This donor was a victim of accidental death without any pathological condition that could affect his reproductive function. The testis tissues were frozen in liquid nitrogen immediately after collection.

RNA extraction

The frozen testis tissues (IR, MR and MH) were ground to fine powder in a liquid nitrogen-cooled mortar, and Trizol reagent (Invitrogen, Carlsbad, CA, USA) was added to the powder with continuing grinding. Total RNA was extracted by Trizol reagent according to manufacturer's protocol. RNA integrity was evaluated by 1% formaldehyde-agarose gel electrophoresis. Those mRNAs were used for both miRNA microarray and qRT-PCR analysis, which was regarded as biological replicates in this paper.

miRNA microarray analysis

miRNAs were enriched from extracted total RNA using mirVana miRNA Isolation Kit (Ambion, Foster City, CA, USA) and labeled with mirVana Array Labeling Kit (Ambion). miRNA microarray assay was performed by CapitalBio Corporation (Beijing, China) based on Sanger miRNAs database miRBase8.2 (<http://microrna.sanger.ac.uk/>, July 2006). The microarray contained 743 degenerated Cy3-labeled oligonucleotide probes generated from 1,172 miRNAs. Double-channel laser scanner LuxScan 10 K/A (CapitalBio) was used for fluorescence scanning, and the image signals were transformed to digital signals using image analysis software LuxScan3.0 (CapitalBio). Signal intensities for each spot were calculated by subtracting local background intensities from total intensities. The miRNA microarray assay was performed twice, and *t*-test was used for statistical analysis. Hierarchical clustering analysis of

miRNA expression was performed using CLUSTER 3.0/TreeView software.

miRNA-specific RT and real-time PCR for miRNA

miRNA-specific RT and real-time PCR were performed on 26 differentially expressed miRNA (> 3-fold difference) shared by IR/MR and IR/MH. Sequences of 22 miRNAs were obtained from Sanger miRNAs database, and six miRNA sequences were Nature predicted. miRNA-specific stem-loop RT primers, miRNA-specific PCR forward primers, universal reverse primers and universal TaqMan probes (FAM/TAMRA labeled) were designed as previously described by Chen *et al* with some modifications [15].

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (*Macaca mulatta*, AY624139; *Homo sapiens*, NM002046) was used as the internal control for miRNA detection. DNase-treated total RNA (2 µg) was reverse-transcribed to cDNA using miRNA-specific RT primers and the RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania). Each 20 µl RT-PCR reaction mixture contained 50 nM RT primers (random primers were used for *GAPDH*). All reactions were performed thrice in a PTC-200 thermocycler (Bio-Rad, Hercules, CA, USA) programed as follows: 25°C for 10 min, 42°C for 60 min, and 70°C for 10 min.

Real-time PCR was performed using an iQCyler thermocycler (Bio-RAD). Each 30 µL PCR reaction mixture included 2 µL cDNA, 1X PCR buffer, 2.5 mM MgCl₂, 0.3 mM of each of dNTPs, 1.5 U DNA polymerase, 1.5 µM forward primer, 0.7 µM reverse primer, and 1.0 µM TaqMan probe. Unless indicated, all reagents used in this procedure were purchased from MBI Company. The reactions were incubated in a 96-well plate at 95°C for 2 min, followed by 45 cycles at 95°C for 15 s, 54°C for 30 s, and 60° for 40 s. All reactions including negative controls were run in triplicates.

Data analysis

Microarray data were analyzed using Significance Analysis of Microarrays software program (SAM, version 2.1, Stanford University, CA, USA). Each gene is assigned a score on the basis of its change in gene expression relative to the standard deviation of repeated measurements for that gene. Genes with scores greater than a threshold are deemed potentially significant.

Real-time PCR data were analyzed using $2^{-\Delta\Delta CT}$ method [11]. The relative amount of each miRNA to *GAPDH* RNA was described using the equation $2^{-\Delta\Delta CT}$, where $\Delta CT = CT_{miRNA} - CT_{GAPDH}$. Relative gene expression was determined by amplification efficiencies derived from the

slopes of the cDNA standard curves using formula $efficiency = 10^{(1/slope)} - 1$ [12].

Bioinformatics analysis of the selected miRNAs

PicTar and TargetScan 4.0 were used to predict target genes for selected miRNAs. Predicted genes whose PicTar scores and TargetScan total context scores exceed their mean scores were selected for functional analysis using NCBI and UCSC databases.

Results

miRNA microarray analysis

Statistical analysis of microarray spot intensities using *t*-test showed good reproducibility and reliability ($P > 0.05$) from the duplicate assays. High reproducibility was also demonstrated by scatter diagrams. The correlation coefficients of IR, MR and MH were 0.9697, 0.9663, and 0.9678, respectively. Hierarchical clustering analyses of miRNA expression showed significant changes (> 3-fold difference) in expression levels for 66 miRNAs shared by IR and MR (Fig. 1a) and 76 miRNAs shared by IR and MH (Fig. 1b).

miRNAs expression

Among the 66 miRNAs (26.4% of all miRNAs detected) of IR/MR, 23 were up-regulated and 43 were down-regulated. Those miRNAs may regulate spermatocytes and spermatids formation and differentiation during spermatogenesis. Among the 76 miRNAs (31.3% of all miRNAs detected) of IR/MH, 28 were up-regulated and 48 were down-regulated. There were 26 miRNAs shared by IR/MR and IR/MH, including nine up-regulated and 17 down-regulated miRNAs.

Furthermore, 15 of the 26 miRNAs were shared by MR and MH, which represented different expression in immature and mature testes of primates. Among the 15 miRNAs, four miRNAs hsa-miR-154, hsa-miR-181c, hsa-miR-181d, and hsa-miR-487b showed high expression in immature testis tissues and low expression in mature testis tissues, and vice versa for the remaining eleven miRNAs, namely hsa-miR-124a, PREDICTED_MIR165, hsa-miR-191*, hsa-miR-296, hsa-miR-34b, hsa-miR-34c, hsa-miR-449, hsa-miR-557, mmu-miR-702, mmu-miR-714, and mmu-miR-715. Those miRNAs were regarded as immature testes (IR) difference with mature testes (MR and MH) and regulated a series of gene expressions which is essential for different types of cell (mainly spermatocytes and spermatids) formation and differentiation during primates spermatogenesis.

Fig. 1 Hierarchical clustering analysis of miRNA expression of primates testis tissues. miRNAs and samples are presented in rows and columns, respectively. Colors indicate relative signal intensities; red and green colors indicate up-regulated and down-regulated miRNAs, respectively; zero and missing miRNAs are respectively shown in dark and light gray colors. **a:** Relative expression levels for the 66 miRNAs that changed significantly (fold changes > 3) are shown in twelve columns corresponding to samples IR and MR. Each oligonucleotide probe was printed in triplicate, and microarray assays were performed twice, resulting in six values for each miRNA. **b:** Relative expression levels for the 76 miRNAs that changed significantly (fold changes > 3) are shown in twelve columns corresponding to samples IR and MH. Clustering analysis was performed using CLUSTER 3.0/TreeView software

The remaining miRNAs are commonly expressed in IR/MR (73.6%) and IR/MH (68.7%). These commonly expressed miRNAs may represent a set of “housekeeping” miRNAs important for regulation of the basic cellular functions in testis tissues. Such as hsa-miR-16 expressed in IR, MR and MH samples. Ro et al. also reported that mir-16 is a “housekeeping” miRNA which was expressed in all of the tissues tested with evenly abundant levels [9].

Real-time PCR for miRNA

Quantitative real-time RT-PCR (qRT-PCR) was used to determine the relative expression levels of the 26 miRNAs shared by IR/MR and IR/MH. The PCR products were detected by 3% agarose gel electrophoresis and showed good specificity, with a single DNA band of the expected size (about 70 bp) (GAPDH was used as internal control,

Fig. 2 Gel electrophoresis of qRT-PCR products. 3.0% high resolution agarose gel electrophoresis was performed with lanes (from left to right) loaded with 50 bp DNA ladder, sample miRNAs hsa-miR-154, hsa-miR-181c, hsa-miR-376b, hsa-miR-124a, hsa-miR-296, hsa-miR-34b, hsa-miR-449, mmu-miR-680 and mmu-miR-709, and internal control GAPDH

124 bp) (Fig. 2). The relative fold changes in miRNA expression as determined by qRT-PCR were similar to those determined by microarray analysis, as shown in the histogram in Fig. 3. The concordance between microarray data and real-time PCR data was identical on the whole.

Putative miRNA target gene prediction

We used PicTar and TargetScan 4.0 prediction programs to predict target genes for the 15 miRNAs (excluding PREDICTED_MIR165) that account for the difference between immature and mature testis miRNA expression.

Fig. 3 miRNA expression was confirmed by qRT-PCR. **a** and **b** Histograms of the relative expression levels of IR/MR and IR/MH. Twenty-six commonly changed miRNAs are listed on the x-axis, and the y-axis refers to the relative expression levels. White and black bars refer to qRT-PCR and microarray results, respectively

As shown in Table 1, the number of predicted target genes varies greatly for different miRNAs. Using NCBI and UCSC databases for functional screening of the putative target genes, we further identified a number of target genes directly involved in spermatogenesis.

The number of target genes predicted by single miRNA varied greatly, ranging from several to hundreds. Three miRNAs (hsa-miR-191*, hsa-miR-487b and mmu-miR-714) have few predicted target genes which can hardly be related to the spermatogenesis. Many miRNAs showed sequence conservation among different species such as miR-181d and miR-296. Conservation of miRNAs among different species suggests that they may bear conserved biological functions. Some miRNAs were cloned from rhesus monkey, such as miR-124a and miR-181c in our studies; those sequence are also conservative.

Discussion

Rhesus monkeys have become the most widely used nonhuman primate species in basic and applied biomedical research due to their abundance and genetical and physio-

Table 1 Putative target genes for 14 miRNAs

miRNA	Sequence	Macaca mulatta	Putative targets associated with spermatogenesis
hsa-miR-154	uagguuauccguguugccuucg	N/A	PPP1CC, PCNA, AQP9, HMGA2
hsa-miR-181c	aacauucaaccugucggugagu	Yes	KPNB1, NR6A1, SOX6, RAD21, CREB1
hsa-miR-181d	aacauucauuugucggugggu	N/A	TIMP3, RNF6, KPNB1
hsa-miR-487b	aaucguacagggucauccacuu	N/A	N/A
hsa-miR-124a	uuaggcacgcggugaaugcca	Yes	QKI, MYO10, SP3, MITF, FGFR2, CDK4, KLF4
hsa-miR-191*	gcugcguuggaauucgucucc	N/A	N/A
hsa-miR-296	aggccccccccuauccugu	N/A	NCALD
hsa-miR-34b	uaggcagugauuagcugauug	N/A	NOTCH1, LGR4, VEZT, MAN2A2, FOXJ2
hsa-miR-34c	aggcaguguaguagcugauugc	N/A	STRBP, LGR4, KLF4, NOTCH1, PPP1CC, GALT, KITLG
hsa-miR-449	uggcaguguauuguuagcuggu	N/A	MECP2, ASB1, BCL2, NOTCH1, CASP2, KITLG, VCL, FOXJ2, INHBB
hsa-miR-557	guuugcacggguggccuugucu	N/A	EIF4G2, STAG2, VCP
mmu-miR-702	ugccccacuuuacccgcuc	N/A	SBF1, MMP14
mmu-miR-714	cgacgaggccggucgucgc	N/A	N/A
mmu-miR-715	cuccgugcacccccgcgug	N/A	KPNB1

The names and sequences of the 14 miRNAs are listed in the first column and second column, respectively. The third column indicates whether the miRNAs have been cloned from *Macaca mulatta*. Genes directly associated with spermatogenesis are presented in the fourth column

logical similarity to humans. According to the draft genome sequence of rhesus monkey completed last year, rhesus monkeys share 97.5% identity with humans at both the nucleotide and amino acid sequence levels [16]. Many miRNAs showed sequence conservation among different mammalian species. To understand miRNA expression during mammalian spermatogenesis, we used miRNA microarray to determine miRNA expression patterns of rhesus monkey (IR and MR) and human (MH) testis tissues. We selected miRNAs with greater than 3-fold change of expression levels and identified 26 miRNAs shared by IR/MR and IR/MH. Further analysis indicated that 15 of the 26 miRNAs may be responsible for the difference between immature and mature testis miRNA expressions. Using a new small RNA cloning method, Ro *et al* identified 141 mouse testis miRNAs, in which 28 miRNAs were testis-specific or testis-preferential. All of the 28 miRNAs showed the highest expression levels in pachytene spermatocytes, or round and elongated spermatocytes, suggesting that late meiotic and haploid germ cells are the main source of miRNA production during spermatogenesis [9]. Our results were consistent with this tendency.

A single miRNAs can directly repress hundreds of direct target genes and downregulate production of hundreds of proteins [17]. Baek *et al* deleted mir-223 in mouse neutrophils and found hundreds of genes directly repressed. The targeting is primarily through seed-matched sites located within favourable predicted contexts in 3'-UTR [18]. We used PicTar and TargetScan 4.0 to predict target genes for the 15 miRNAs. Some miRNAs have a number of

predicted target genes, such as hsa-miR-218 have over four hundreds predicted target genes. Some of these genes may be involved in spermatogenesis during the development of germ cells. Some genes were testis-specific and can be sorted into several groups based on their expression patterns. One important group of genes encode for transcription factors (TFs) such as *CREB1* (cAMP responsive element binding protein 1) [19], *FOXJ2* (Forkhead box J2) [20], *HMGA2* (High mobility group AT-hook 2) [21], *KLF4* (Kruppel-like factor 4) [22], *MITF* (Microphthalmia-associated transcription factor) [23], *NOTCH1* (Notch gene homolog 1) [24], *SOX6* (SRY-box containing gene 6) [25] and *SP3* [26]. miRNAs preferentially regulate positive regulatory motifs, highly connected scaffolds and downstream network components such as TFs, and regulate signaling networks in multiple ways [27]. Zhou *et al* showed that interacting TF-miRNA, TF-TF or miRNA-miRNA pairs tend to regulate very large numbers of genes [28]. Another group consists of apoptosis-related genes such as *BCL2* (B-cell leukemia/lymphoma 2) [29] and *CASP2* (Caspase 2) [30].

It is estimated that about 5,000 human genes (~ 20% of all human genes) are subject to miRNA regulation. Ever since its discovery, miRNAs have been regarded as negative post-transcriptional regulators of gene expression. However, recent work by Vasudevan *et al* showed that miRNAs down-regulate gene expression when cells are dividing but up-regulate gene expression when cells are quiescent [31]. In our study, four miRNAs, hsa-miR-154, hsa-miR-181c, hsa-miR-181d and hsa-miR-487b were highly expressed in immature primates testis tissues but not mature testis tissues. Some of the predicted target genes

for these miRNAs might be directly involved in spermatogenesis. *AQP9* (Aquaporin-9) is a new member of the aquaporin family of water-selective channels and predicted by hsa-miR-154. *AQP9* is strongly expressed in the plasma membranes of Leydig cells in rat testis and adult dog testis. Studies showed that *AQP9* may be involved in the early stages of spermatogenesis and in the secretion of tubule liquid [32, 33]. *RNF6* (ring finger protein 6) is predicted by hsa-miR-181d and show increased expression during meiosis. *RNF6* is predominantly expressed in Sertoli cells and pachytene spermatocytes, and as a regulator of transcription it has been shown to increase transcription levels in maturing Sertoli cells [34].

We also identified eleven miRNAs that showed high expression in mature primate testis tissues but not immature testis tissues. Some putative target genes regulated by those miRNAs may play roles in differentiation of germ cells and spermatogenesis. *NOTCH1* is predicted by hsa-miR-34b, hsa-miR-34c and hsa-miR-449 and can be recognized in the vacuoles of the Golgi of primary spermatocytes and the acrosomes of elongated spermatids with electron microscopy. *NOTCH1* is important for the survival and differentiation of germ cells in the rat testis but fails to express in the testes of patients with spermatogenic maturation arrest [24]. *Sp3* is predicted by hsa-miR-124a and activates the testis-specific histone H1t promoter through the H1t/GC-box in pachytene primary spermatocytes and early spermatids [26]. *Bcl-2* is predicted by hsa-miR-449 and mediates spermatogonial apoptosis, a crucial part of normal mammalian spermatogenesis. It was found that transgenic mice expressing high levels of the *BCL2* proteins in the male germinal cells exhibited highly abnormal adult spermatogenesis accompanied by sterility [29].

Some of the 15 miRNAs whose expression differ between primates immature and mature testis tissues have been cloned and sequenced from many species. hsa-miR-154 has been cloned from *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Pan troglodytes*, *Gorilla gorilla*, *Pongo pygmaeus*, *Macaca nemestrina* and *Pan paniscus*. miR-124a and miR-181c have been cloned from rhesus monkey. Many miRNAs are conserved between rhesus monkey and human, making it possible to clone new miRNAs from rhesus monkey based on conserved sequences of human miRNAs. Prediction of target transcripts often yields false targets due to imperfect base pairing and the short length of binding sites. Examining the expression of predicted target mRNAs may provide clues on whether the miRNA-mediated regulation is direct or indirect. Defining the targets and mechanisms whereby miRNAs, likely in association with many of the numerous RNA-interacting testicular proteins, modulate post-transcriptional gene expression in the testis promises to reveal a new level of cellular regulation in gamete differentiation. In summary,

our data demonstrate that miRNAs are crucial for regulating gene expression in spermatogenesis. Future studies to analyze miRNA expression patterns and their effects on target mRNA expression may help to elucidate the precise role of miRNAs in spermatogenesis.

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