microRNA profiling in three main stages during porcine spermatogenesis

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Abstract

Background Spermatogenesis is an intricate biological event wherein an undifferentiated spermatogonium develops into mature sperms. MicroRNAs are a type of single strand small non-coding RNA molecule and are implicated in the regulation of many crucial pathways during cell proliferation, apoptosis, and differentiation.

Zonggang Luo and Yingkai Liu contributed equally to this work.

Capsule ZGL carried out the molecular genetic studies, participated in the sequence alignment, and revised the manuscript. YKL participated in the study design and drafted the manuscript. LC carried out the RT-PCR and data analysis. ME participated in the experiment.

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Method Here, we present a comprehensive comparison of miRNA expression profiling in three main stages during porcine spermatogenesis using high-throughput sequencing.

Results We built three small RNA libraries for the testis, the epididymis and the ejaculated sperm from a Landrace boar, and in total obtained 3821 precursor hairpins encoding for 4761 mature miRNAs, of which 23 are miRNA*. Notably,

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940 precursor miRNAs produced both the 5'- and 3'- strands as sister pairs, indicating the distinctive expression patterns of germ cell miRNAs. Additionally, 418 out of 710 co-expressed miRNAs were identified as being differentially expressed between libraries ($P < 0.001$). Apart from the sexual specific X chromosome, many miRNAs were found to be located on chromosome 12, which may play potential roles in spermatogenesis according to the result of synteny analysis with human and mouse. The Gene Ontology and KEGG pathway analysis revealed that the target genes of co-expressed miRNAs were highly involved in the cell cycle process, metal ion binding, modification of plasma membrane, and the p53 signal pathway.

Keywords microRNAs · Spermatogenesis · Deep sequencing · Testis · Epididymis · Sperm

Introduction

Spermatogenesis is a complex process in which the diploid spermatogonia go through a series of biochemical, physiological and morphological changes in order to form haploid spermatozoa [1]. Most of these processes, such as nuclear chromatin condensation and flagellum development, happen in the testis. The testis functions to produce immature spermatozoa, which lack motility and therefore the ability to fertilize. The epididymis, wherein the maturation of spermatozoa takes place, contains many specific proteins required by post-gonadal modification [2]. This organ functions in sperm storage and ejaculation [3]. Before the spermatozoa become capable of fertilization, each step from the testis to the oviduct of the female makes finite contributions to their dynamic and functional capacity.

Sperm RNA, due to its limited amount and special anatomical position, has been considered as the residue of spermatogenesis without functions for a long time [4–6]. Until recently, it was reported that a number of mammalian sperm RNAs participate in the regulation of spermatogenesis and fertilization [7]. Some sperm specific RNAs can even be delivered to the ooplasm, indicating the unique paternal contribution to the zygote [8–11]. Besides sperm RNA populations, microRNAs (miRNAs) are also of interest due to their key roles in the regulation of gene expression. It has been estimated that about one third of human genes are regulated by miRNAs, which are highly conserved across species [12]. These small endogenous non-coding RNAs are usually ~22 nucleotides (nt) in size, and they have been reported to play critical roles in cellular processes [13], embryo development [14], the immune system [15], and diverse diseases [16].

Focusing on spermatogenesis, the latest discoveries in mouse and humans indicate that genes related to zygote

development are regulated by sperm miRNAs, together with piRNAs and tRNA-derived small RNAs [17, 18]. In the male mice germline, the knockout of the enzyme *Dicer*, which plays essential roles in the production of miRNA, would lead to embryonic lethal [19]. Novotny and colleagues found that miR-17–92 cluster down regulates *E2F1* gene, protecting meiotic cells from apoptosis during normal spermatogenesis [20]. Yu *et.al* suggest miR-122a regulate post-meiotic germ cells by base pairing the 5' region to the complementary site in *Tnp2* mRNA [21]. miR-34b was identified to play a potential role in the differentiation of male germ cells when comparing adult testis and pre-puberal testis [22]. Using luciferase assay and Western blotting, Liu *et.al* identified that miR-34c has an effect on B-cell leukemia/lymphoma 2 (*Bcl-2*) expression in the zygotes [23]. Based on the facts above, it indicates that miRNAs play essential and complex roles during spermatogenesis and the exploration of their unknown functions is in urgent need.

Despite the increasing numbers of sperm miRNAs that have been discovered across species, detailed levels of specific miRNAs involved in the spermatogenesis process are yet to be determined. In this paper, we focus on the three main steps during porcine spermatogenesis and explore the miRNA expression of the testis, the epididymis, and the ejaculated sperm using deep sequencing. The characteristic organ-specific profiles are elucidated, which may provide guidance for further reproduction research and economic breeding.

Materials and methods

Animal ethics statement

All of the research, including the animal component, was conducted based on the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised in June 2004). The experiment was approved by the Institutional Animal Care and Use Committee in the College of Animal Science and Technology, Southwest University, Chongqing, China.

Sample collection

The fresh ejaculated sperm was collected from a healthy male Landrace pig (2-year-old) using the gloved hand technique. The sperm quality was evaluated under a microscope requiring more than 75 % of spermatozoa with good motility and morphological appearance. The sperm pellets were washed twice in PBS, centrifuged, and the supernatant was removed. 0.1 % SDS (sodium dodecyl sulfate) and 0.5 % Triton X-100 were applied for the removal of the somatic cells [24]. The purity of the sperm sample is checked more than 80 % before

its storage in freezer (-80°C) until use. The testis and the epididymis were collected from the same boar, and were immediately frozen in liquid nitrogen and then placed in freezer (-80°C) for storage.

Small RNA library construction

Small RNAs from each tissue were extracted by using the mirVanaTM miRNA isolation kit (Ambion, Austin, TX, USA) following the manufacturer's procedure. The quality and purity of the total RNA was measured by using a NanoDrop ND-1000 spectrophotometer (Nano Drop, DE, USA) with the ratio >2.0 at 260 and 280 nm. Then the Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, USA) were hired to ensure the integrity of the experimental RNAs, with the RIN number more than 6.0. Qualified RNA molecules were obtained after the above tests and were prepared for small RNA library construction. For each tissue, 45 μg total RNA was used for both library preparation and sequencing. The 10–40 nucleotides short pieces were isolated by polyacrylamide gel electrophoresis (PAGE) to complete the ligation with the proprietary adaptors (Illumina, San Diego, CA, USA). Subsequently, the small RNAs were converted into cDNAs by RT-PCR and were sequenced by the Genome Analyzer GA-2 (Illumina) according to the manufacturer's recommended protocol for small RNA sequencing.

In silico analysis of the sequencing results

As described in Liu's previous study of miRNA profiling using deep sequencing [25, 26], the bioinformatics pipeline was carried out with the newest version with certain improvements. All the raw reads were modified by the combination of Illumina's Genome Analyzer Pipeline software along with self-developed ACGT-102 miR program (LC Sciences). The "mappable reads" were generated after a series of additional filters including other known classes of small RNAs followed by the acceptance criteria derived from the statistics of mammalian miRNAs in miRBase 19.0 (July, 2012). In order to ensure a high reliability of the sequencing results, only reads with more than three copies were applied for further mapping analysis to the pig genome (~ 3.02 Bbp) (Sscrofa10.2, GCA_000003025.4) by using NCBI Local BLAST.

To classify the functions of the reported sequences, we combined PicTar ([http://pictar.mdc-berlin.de/cgi-bin/PicTar_Vertebrate.cgi](http://pictar.mdc-berlin.de/cgi-bin/PicTar Vertebrate.cgi)) and TargetScan (version 6.2, <http://targetscan.org>) online searching software for target mRNA prediction. Subsequently, the overlaps of these two tools were applied for the functional annotation by Gene Ontology (GO) and KEGG pathway analysis in the Database for Annotation, Visualization and Integrated Discovery (DAVID).

qRT-PCR for validation

Total RNA was reverse transcribed to cDNA using the oligo (dT) and poly(A) polymerase provided in the PrimeScriptTM miRNA RT-PCR Kit (TaKaRa, Dalian, China). The SybrGreen-based qRT-PCR approach was performed to validate the sequencing result, and the primer sequences were listed in Supplementary file 8: Table S6. The *18S rRNA* and *Met-tRNA* were set as controls for all amplifying reactions and each reaction was conducted in triplicate. The quality of the amplified products was tested by 1 % agarose gel, and then the $\Delta\Delta\text{Ct}$ method was used to calculate the relative expression levels of each miRNA.

Results and discussion

Summary of the sequencing results in three libraries

In order to identify miRNA populations in the main steps during porcine spermatogenesis, we built up three small RNA libraries for the testis, the epididymis, and the ejaculated sperm using solexa high-throughput sequencing. After filtering out low quality data which did not meet the criteria previously developed [25, 26], we obtained 5.03, 9.83, 7.26 M clean data from each library, which are referred to as "mappable reads". As in other tissues, various kinds of small non-coding RNAs as well as miRNAs may also contribute to the gene regulation during the spermatogenesis process. In our sequencing results, some reads were mapped to certain other known classes of small RNAs (Supplementary file 1: Table S1), which were eliminated from the analysis.

Fig. 1 Length distribution and frequency (%) of mappable reads. The frequency value indicates the counts of the total sequenced sequence and is calculated by the ratio of count numbers at each stage and the total sequencing results. The testis (orange line) and the epididymis (blue line) showed the most abundant value at 22 nucleotides (nt) followed by 21 and 23 nt, whereas the ejaculated sperm (grey line) reaches the peak value at 20 nt

The length distribution of the mappable reads is summarized in Fig. 1, in both testis and epididymis libraries, the peak value was shown at 22 nt, followed by 21 and 23 nt, which match well with the typical product size of the Dicer enzyme [27, 28]. Notably, small differences were shown in the ejaculated sperm library, where the overwhelming majority of the miRNAs were less than 20 nt in length. These smaller size miRNAs from the ejaculated sperm also showed lower expression levels compared to those in the other two tissues.

Based on previous studies by Li *et al* [29, 30], we divided the mappable reads into three groups with progressively decreasing confidence: (1) porcine known miRNAs (Supplementary file 2: Table S2.1), with both mature miRNAs and the precursors mapped to the pig genome; 212 porcine known pre-miRNAs generated 392 mature miRNAs. Additionally, 23 unknown miRNA*s were detected in our library due to the development of sequencing methods; (2) porcine novel miRNAs (Supplementary file 2: Table S2.2), 291 pre-miRNAs that have not been mapped to known porcine pre-miRNAs, but are homologous to other known miRBase mammalian pre-miRNAs, which confirmed that most of the miRNAs are highly conserved across various species [31]. These precursors produced 291 mature miRNAs; and (3) porcine candidate miRNAs (Supplementary file 2: Table S2.3), with predicted RNA hairpins in the genomic loci that express identical mature sequences, that consisted of 3382 candidate pre-miRNAs coding for 4078 mature miRNAs.

Expression of both 5' and 3' strand in germ cell miRNAs

miRBase 19.0 (August, 2012) reported 280 pre-miRNAs encoding 326 known porcine miRNAs. We found 392 porcine known miRNAs, of which 169 shared the same sequence with reported miRNAs, 200 were isomiRs with 1~2 mismatches, and 23 were newly discovered (Supplementary file 2: Table S2.1). Obviously, most of these novel porcine known miRNAs were miRNA*s corresponding to their previously discovered strand.

Given the rules of thermodynamic stability, the ~70 nt stem-loop precursor miRNA is believed to produce only 5' strand or 3' strand mature miRNA with more thermodynamic stability then destroy the other one, which is usually regarded as miRNA* [32]. In most cases, it is difficult to detect miRNA* because of its rapid turnover and time-specific expression pattern. However, in a small portion of tissues, both miRNA and miRNA* from the same precursor with similar thermodynamic stability can be generated and incorporated into the RNA-induced silencing complex (RISC) at the same time [32]. Ro *et al* found that out of 969 mouse and human miRNAs registered in the miRBase 9.2 (May, 2007), 234 miRNAs (117 pairs) contained both the 5'- and 3'- strands as sister pairs. These paired miRNAs are expressed in certain tissues like testis [33]. They also found out that these sister

pairs might function varying efficiency with different target genes. Take miR-30e-5p and -3p as an example, miR-30e-5p showed its preferentially accumulation in stomach with the validated target genes *Mrps30*, *Npffr2* and *Spata19*; however, the sister mir-30e-5p selected to accumulate in spleen with the target genes *Akap14*, *Tshb* and *Sap30* [33].

As shown in Fig. 2, from the total 4761 miRNAs detected in our three libraries, 1447 are 5'- strand miRNAs and 1434 are 3'- strand miRNAs. The remaining 1880 (940 pairs) miRNAs are sister pairs with strands derived from the same precursor, accounting for one third of the total sequenced mature miRNAs. Our results confirmed that the co-expression of miRNAs and their corresponding miRNAs* coming from the same precursor is one of the characteristics of the testis-expressed miRNAs [34]. Despite their co-accumulation, these sister paired miRNAs showed unique expression profiles (Supplementary file 3: Table S3.1), suggesting that they are independent of each other and have their own stage-specific expression levels.

The high rate of detection of paired miRNAs in gonad-specific libraries indicate their potential roles in the regulation of gonad development as well as spermatogenesis, and more attention should be paid to these sister pairs in future studies.

Chromosome distribution and miRNAs on the X chromosome

The difference between sex chromosomes and autosomes is one of the essential facts we need to take into account when considering the sex-determination system. In our study, we calculated the genomic location distribution of the miRNAs across the porcine chromosomes. As shown in Fig. 3a, the X chromosome contained 0.96 (average of three libraries) miRNA per 1 kb, while the average density of the rest autosomes was 0.92 miRNA per 1 kb. This slight difference between the X chromosome and other autosomes may indicate the essential roles of miRNAs on chromosome X, similar results were also found in previous reports [35]. Recent studies demonstrated that some miRNAs on chromosome X were expressed in a testis-preferential pattern [36], which may play potential roles in diverse functions during spermatogenesis [37]. In addition, the expression levels of these X-derived miRNAs also showed higher expression levels than most of the ones derived from autosomes [38].

Apart from the sex-determination X chromosome, another autosome, chromosome 12 showed an abundance of miRNA expression (1.82 miRNA per 1 kb), and the same occasion happened in Li's sequencing results about miRNAs in the testis and ovary [26]. We wondered whether the high miRNA density was related to the coding genes, thus we downloaded the data for coding genes of each chromosome from Ensembl (http://asia.ensembl.org/Sus_scrofa/Location/Genome). Interestingly, there was a relatively high correlation ($r=0.71$, Fig. 3b) between the coding gene

Fig. 2 Expression profiles of the 5'-strand and the 3'-strand miRNAs. The pie chart shows the numbers and the percentage of the precursor miRNAs (grey) and the mature miRNAs (orange) detected in our results.

density and the miRNA density, indicating there could be some relationship between these two and further study is required. In order to identify the potential functions of these loci-specific miRNAs, we tried to but failed to find out the bibliographies that reported on miRNAs from porcine chromosome 12 (chr 12). For this reason, we did Synteny analysis by using online database Ensembl 2013 (<http://www.ensembl.org>) [39], which calculated syntenic regions between two species based on whole genome alignments. As shown in Fig. 4, the Human chr 17 and the mouse chr 11 were separately compared with the porcine chr 12 and showed high synteny between the two species. Based on this result, we explored the functions of genes located on these two chromosomes. Surprisingly, the human chr 17 and the X chromosome showed similar alpha satellite hybridization pattern [40], and there are also genes related to growth hormone on human chr 17 [41]. Moreover, it was reported that six mutations on mouse chr 11 could cause reduced sperm number in male sterility (infertility *inf2*, -4, -5, -6, -8, -9) [42]. All these facts suggested that miRNAs on porcine chr 12 may have potential functions in regulating genes during gonad development.

Highly expressed miRNAs

In mammals, the expression level of a single miRNA exhibits a very wide range (from several to hundreds of thousands), but only a few of them showed their abundance. As summarized in Fig. 5, the top 15 expressed miRNAs accounted for 73.35 % (testis), 72.25 % (epididymis) and 66.53 % (sperm) of the total mappable reads in each library, resulting in a total of 26 top expressed miRNAs across the three tissues. Interestingly, the first eight miRNAs on the left side of Fig. 5 (shown in light orange bar) were the top co-expressed miRNAs in the testis and epididymis libraries, however, a relatively lower expression level of these was exhibited in the ejaculated sperm library ($P < 0.01$). Similar results were obtained in previous studies that showed hsa-miR-143, hsa-miR-125a, hsa-miR-125b and hsa-let-7 family members represented the most frequent, with high or relatively lower expression level throughout the whole lifespan [43].

Out of the 3821 pre-miRNAs detected by deep sequencing, 940 precursors produced both 5'- and 3' strands as sister pairs, which accounts for more than one third of the total sequenced sequences

miRNAs from let-7 family are predicted to target *HMGA2* [44, 45], which is presumed to serve an essential function in spermatogenesis, as *HMGA2* null mice produced no mature sperm [46].

In Fig. 5, the three light blue bars, representing miR-10b, miR-26a and miR-191, were the co-expressed miRNAs in three libraries. These three small RNAs shared a similar expression level in each tissue, and quantitative real time RT-PCR was applied to validate the results (Supplementary file 5: Fig S1). To further explore the functions of these three miRNAs, we combined two software programs, PicTar (<http://pictar.mdc-berlin.de/>) and TargetScan 6.2 (<http://targetscan.org/>), to predict their target genes, and only the overlapping areas of the results of these searches were set as the final target mRNAs. Subsequently, Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7) was used for Gene Ontology (GO) and (KEGG) pathway analysis. The most significant GO term with the smallest P value ($P = 0.001$) was embryonic organ development, in accordance with the fact that spermatogenesis is a continuous cell division and regenerative process. Furthermore, many GO terms indicated that these miRNAs were related to the cell cycle process (Supplementary file 6: Table S5.1), corresponding to the biological process including mitosis of spermatogonia and meiosis of spermatocytes to complete spermatogenesis [35]. Moreover, metal ion binding and cation binding are two most enriched GO terms, with 29 (35.37 %, $P < 0.05$) predicted genes participating in the progress. Ca^{2+} and its ion channel is reported to be one of the key factors to control sperm motility, the acrosome reaction and flagellar-beat mode (including hyperactivation), and plays a very important role in capacitation [47–51]. Abnormal levels of the ion signal pathway may lead to sperm activity disability [52].

Additionally, the seven miRNAs indicated by the light green bars in Fig. 5 showed a preferential expression mode in the sperm library, where they were from two to hundred fold higher in abundance than shown in the other two libraries ($P < 0.01$). It is reported that mmu-miR-449a and hsa-miR-449a were the most abundant in mouse and human gonad tissues [53]. Recent studies indicate that miR-16 and miR-34 were sperm-preferentially expressed and play important roles

Fig. 3 a Chromosome distribution of the sequenced miRNAs. Densities were calculated by dividing the number of miRNAs on individual chromosome by the length of nucleotides on the corresponding chromosome; **b** correlation between the coding gene density and the miRNA density ($r=0.71$). Chr 12 is labeled in the figure with the highest coding gene density and miRNA density

in cell cycle process during spermatogenesis [54]. Target prediction of these seven sperm-bias miRNAs produced a total of 546 target genes corresponding to 523 unique mRNA-miRNA interactions, and 266 GO terms and 21 KEGG pathways (Supplementary file 6: Table S5.2). “Pattern specification process” ranks the top of the DAVID list with the smallest P value ($P=6.48E-05$), suggesting that a potential specific mode, which may be essential to the acrosome reaction and post-sperm development. Notably, GO

terms relating to the plasma membrane were also highly abundant ($P=5.59E-04$), in accordance with the fact that sperm cells have unique membranes compared to those of somatic cells, which have different plasma membrane specializations with the same goal to complete the fusion with the oocyte [55]. The early papers demonstrate that sperm plasma membrane play important roles in mammalian fertilization, being involved with a number of processes including the release, and the modification and adsorption of proteins and

Fig. 4 Synteny analysis between porcine chromosome 12 and human chromosome 17 (a) and mouse chromosome 11 (b), respectively. The syntenic regions were obtained from the online database Ensembl 2013, and are shown as colored blocks. Lines connected between blocks indicate regions with the same (black) or the opposite (brown) orientation. The small red boxes mark the gene of interest and its homologue (see Supplementary file 4)

lipids [56]. The only significant pathway that was identified by KEGG analysis was the “p53-signaling pathway”, resulting from the fact that miR-34 is regulated by its upstream factor, the tumor suppressor protein p53 [57, 58]. This miRNA has also been reported to take part in the negative control of the cell cycle [59], cell cycle arrest [60], senescence [61], and apoptosis [62].

Notably, despite absence in the most frequently expressed miRNAs in any of the three libraries, several miRNAs have been identified to have a gonad-specific pattern during spermatogenesis with a high expression level (Supplementary file 3: Table S3.2). For example, miR-122a is predominately expressed in late-stage male germ cells and it represses the transcription of transition protein 2 (*Tnp2*) [21], a marker for round spermatids, suggesting that miR-122a functions in post-

meiotic germ cells. miR-29 is reported to be gonad abundant and maintain a high expression level in the adult stage, especially in the epididymis tissue [63–65], and it is usually regulated by androgen during the development of the epididymis [66]. Another example is miR-17, which is up-regulated to avoid spermatozoa apoptosis during the development of sperm [20], usually maintains a high level in the gonad-related tissues.

Differential expressed (DE) miRNAs

As shown in Fig. 6, out of 4761 sequencing results, 710 (14.91 %) miRNAs were co-expressed in the three libraries, and 764 (16.05 %), 1416 (29.74 %) and 784 (16.47 %) were testis-, epididymis-, and sperm-specific miRNAs,

Fig. 5 Top 15 unique miRNAs with the most abundant expressions in three libraries. The top 15 expressed miRNAs were chosen from each library, partially overlapped and made a total of 26 top expressed miRNAs in all three tissues. The circles (blue for testis, red for epididymis and orange for sperm) indicate the count number of corresponding miRNA for each individual and the bar indicates the average expression level of the three libraries. The IDEG6 program was

applied to the normalization calculation and all 26 miRNAs showed differential expression between each combination of two libraries ($P < 0.001$, see Supplementary file 3: Table S3.2). The light orange bar represented miRNAs expressed in almost all tissues through lifetime; the light blue bars meant these miRNAs showed a very high expression level in all three tissues; the green bars showed miRNAs which had a higher expression level in sperm compared with the other two tissues

Fig. 6 Venn diagram showing the shared miRNAs from each library. In total, 710 mature miRNAs were co-expressed across the three libraries, and 418 of them were identified differentially expressed ($P < 0.001$, in dash circle) by IDEG6 program

respectively. Intriguingly, from the testis, the epididymis to the ejaculated sperm in order, the number of overlap miRNAs between every two libraries decreased, which is in accordance with the physiological relations between every two tissues. In order to determine the differential expression (DE) levels between libraries, the IDEG6 program [67] was used for the normalization calculation. A unique miRNA was considered to be significantly differentially expressed only when both Fisher exact test and Chi-squared 2×2 test resulted in a P value < 0.001 [26, 30]. Among the 710 co-expressed miRNAs, 418 were differentially expressed miRNAs (Supplementary file 3: Table S3.2, Supplementary file 7: Fig S2), including the top 26 expressed small RNAs. It is worth noticing that the miR-34 abundance in the sperm is 922.16 times ($P < 0.001$) and 57 times ($P < 0.001$) greater than in the epididymis and the testis, respectively. In addition, another miRNA miR-16 showed 15.15 times ($P < 0.001$) and 24.88 times ($P < 0.001$) greater expression levels when compared to the two immature-sperm libraries, respectively (Fig. 5). It is reported that both miR-34 and miR-16 are up-regulated during the development of the germ cell and can negatively regulate the cell cycle progression [21, 58, 68, 69]. Spermatogenesis is a process where immature spermatogonia develop the ability of fusion with the oocyte, and the arising expression level of these two miRNAs from testis to sperm confirmed the previous discovery.

The miR-34 family is an evolutionarily conserved family containing three members: miR-34a, miR-34b, and miR-34c, which are induced upon p53 activation in multiple cell types. *BCL2* and *MYCN* were identified to be targeted by miR-34a as the tumor cell suppressor. miR-34b had a modest effect but miR-34c can obviously inhibit cells growth [70]. The predicted miR-34 target genes encode for proteins that are involved in cell cycle regulation, apoptosis, and growth factor signaling [70, 71]. In our study, both miR-34b and miR-34c ranked in the top 15 expressed miRNAs in the sperm library, indicating their potential roles in the later period of the sperm development. Barad and his colleagues compared the sperm miRNAs

of the adult and the pre-puberal testis, and indicated that miR-34b is an essential factor in the differentiation of the human male germ cells [72]. Bouhallier *et.al* used Northern blot and in situ hybridization to prove that miR-34c is mainly expressed in germ cells especially in round spermatids [73]. According to our result, the sperm miR-34b showed 3468 ($P < 0.001$) and 138 ($P < 0.001$) times greater and the miR-34c was 922 and 57 times greater ($P < 0.001$) than that of testis and epididymis library, respectively, which indicated their initial roles along with the maturation of the germ cells. It has been experimentally confirmed that *TGIF2* is a target gene of miR-34c, and its down-regulation largely enhanced the TGF β pathway signal which plays a major role in round spermatids and pachytene spermatocytes during spermatogenesis [74–76]. The NOTCH signaling pathway controls various events related to cell-fate specification. The down-regulation of *NOTCH2* by miR-34c is relevant for the differentiation process [77, 78]. More importantly, Liu *et.al* using qRT-PCR method has shown that only the sperm, rather than the oocytes and zygotes, contained precursor miR-34c. They also demonstrated that miR-34c is important for the first cell division via modulation of *Bcl-2* expression [23]. Above all, this evidence suggests that several germ cell specific miRNAs, especially some sperm highly expressed miRNAs, not only play important roles in maintaining their functions of producing a functional sperm during spermatogenesis, but also can be delivered into oocyte in order to complete the fertilization process, which would lead to a normal embryo development.

Conclusions

Since artificial insemination is widely used in commercial livestock reproduction, the quality of the sperm is one of the most important factors affecting the success rate of the fertilization event. It has been widely shown that sperm from different anatomic sites show wide variation in shape and function. For example, the sperm from the epididymis and the ejaculated fresh sperm can be fused with the oocytes [79], and sperm from the epididymis are less sensitive to cold shock and is easier to freeze for storage [80]. Nevertheless, the details of these sperm production channels and the potential molecular mechanisms involved remain unknown. In our study, we put the three major parts during spermatogenesis together and compared their miRNA expression levels by solexa deep sequencing. Our results showed the enrichment of gonad-specific miRNAs in each physiological region during germ cell development, which might show their potential roles in the regulation of the production of a functional sperm. We believe all these results will provide a basic reference for the research of porcine reproduction, and further can be even used in the selection of boars with good reproductive performance and will result in economical improvements in pig industry. Of equal

importance, the understanding of spermatogenesis at miRNA levels will also help reveal the regulation of posttranscriptional-mediated genes in mammalian reproduction.

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