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Comparison of microRNA Transcriptomes Between Immature and Mature Porcine Testes

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Abstract: High-throughput sequencing of two small RNA libraries derived from immature (20 days old) and mature (210 day old) porcine testis samples yielded over 20 million high-quality reads. Researchers detected 461 mature microRNAs (miRNAs) encoded by 277 precursor (pre)-miRNAs of which 428 were unique. In total, 303 unique miRNAs of (428, 70.79%) were differentially expressed between immature and mature porcine testes. Compared with immature testis, 95 unique miRNAs were up-regulated and 208 unique miRNAs were down-regulated in mature testis. Strikingly, researchers found that most miRNAs and differentially expressed miRNAs were preferentially located on the X chromosome which implied their crucial roles in the sex-determination system. Furthermore, GO and KEGG analyses of the target genes that were predicted from the highly abundant differentially expressed miRNAs between mature and immature porcine testes illustrate the likely roles for these miRNAs in spermatogenesis. The study indicates that miRNAs are extensively involved in spermatogenesis and that unraveling miRNA functions in the testis will further the understanding of regulatory mechanisms of mammalian spermatogenesis and male infertility treatment.

Key words: High-throughput sequencing, microRNAs, transcriptome, porcine, testis, spermatogenesis

INTRODUCTION

Each testis contains many seminiferous tubules where germ cell differentiation takes place and a population of diploid stem-cell spermatogonia lie on the basement membrane of the tubule. Spermatogenesis is the development and differentiation of germ cells that take place in the seminiferous tubules of the mammalian testis which is a strictly regulated process through which diploid germ cells proliferate and differentiate intohaploid spermatozoa (Cooke and Saunders, 2002). A novel mechanism of microRNA (miRNA)-mediated post-transcriptional control has lately come to be regarded as an important regulator of reproductive processes (Ogorevc *et al*., 2011; McIver *et al*., 2012).

miRNAs are small, endogenous, non-coding, regulatory RNAs that play key roles in a broad range of biological processes (Ambros, 2004; Bartel, 2004; Pencheva and Tavazoie, 2013) including development, differentiation, tissue morphogenesis, disease and spermatogenesis (Hatfield *et al*., 2005; Lian *et al*., 2010). Previous studies showed that numerous miRNAs are exclusively or preferentially expressed in the testis or male germ cells (Ro *et al*., 2007; Yan *et al*., 2007; Smorag *et al*., 2012). In addition, miRNA expression patterns are also reported to be different between immature and mature testes (Luo *et al*., 2010; Lian *et al*., 2012). Recent studies showed that miR-383 expression is down-regulated in the testes of infertile men with Maturation Arrest (MA) (Tian *et al*., 2013) and that miR-20 and miR-106a regulate spermatogonial stem cell renewal at the post-transcriptional level via targeting Signal Transducer and Activator of Transcription 3 (STAT3) and G1/S-specific cyclin-D1 (CCND1) (He *et al*., 2013a, b).

To reveal the regulatory role of miRNAs in testes development and spermatogenesis, researchers employed high-throughput sequencing technology to compare the miRNA transcriptome between sexually immature and mature porcine testes. Researchers found that most miRNAs were Differentially Expressed (DE) between immature and mature testes and that both miRNAs and DE miRNAs were preferentially located on the X chromosome which implied the important roles of X-linked miRNAs and DE miRNAs during spermatogenesis.

MATERIALS AND METHODS

Animal ethics statement: All research involving animals was conducted according to the regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China,

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revised in June 2004) and approved by the Institutional Animal Care and Use Committee in the College of Animal Science and Technology, Sichuan Agricultural University, Sichuan, China under permit No. DKY-S20112716.

Sample collection and total RNA isolation: The testes were obtained from three 20 days old and three 210 days old male Tibetan pigs. All samples were frozen in liquid nitrogen immediately and stored at -80°C. Total RNA was extracted using the mirVana™ miRNA isolation kit (Ambion, Austin, USA) according to the manufacturer's protocol. The integrity of total RNA was determined by the Agilent Technologies of Bioanalyzer 2100 and RNA 6000 NanoLabchip kit (Agilent, CA, USA) with RIN number >6.0.

Small RNA library construction and sequencing: For small RNA library construction, total RNA isolated from the testes of three 20 days old male Tibetan pigs (sexually immature) and three 210 days old male Tibetan pigs (sexually mature) were pooled and prepared according to the Illumina EAS small RNA Sample Prep Protocol. In general, approximately 15 ug of small RNA-enriched total RNA was prepared for high-throughput sequencing. In brief, high-throughput sequencing was performed as following successive step: for each library, the small RNA ranged from 14-40 nt were purified by Polyacrylamide Gel Electrophoresis (PAGE) and ligated with proprietary adaptors (Illumina). Then, the modified small RNA was reverse transcribed and amplified by RT-PCR. Finally, the enriched cDNA were sequenced on Genome Analyzer II (GAI, Illumina, San Diego, CA, USA) according to manufacturer instructions.

Analysis of high-throughput sequencing data: The raw reads were performed using a proprietary pipeline, ACGT101-miR Program (Illumina) and followed a series of additional filters (Li *et al*., 2010, 2011). After trimming off the sequencing adapters and junk reads, the resulting reads was successively filtered by read length, sequence component. Then, the retained reads were searched against the NCBI, Rfam and Repbase database to remove porcine known classes of RNAs (i.e., mRNA, rRNA, tRNA, snRNA, snoRNA and repeats). The sequencing reads survived from above strict filter rules were deemed as high-quality reads.

MiRNA differential expression analysis: To analyze the differential miRNA expression between two libraries (sexually immature and mature porcine testes), first the expression of miRNAs in two samples were normalized by total high-quality reads. The p-value were calculated from the normalized expression using program IDEG6. Fold-change were also calculated from the normalized expression (If the normalized expression of a given miRNA is zero, the expression value was modified to 0.01). A unique miRNA is considered to be differentially expressed when it simultaneously obtain $p<0.001$ under three statistical test (Audic-Claverie test, a Fisher exact test and a Chi-squared 2×2 test with the Bonferroni correction to adjust for pair-wise comparison).

Prediction and functional annotation of miRNA target genes: The potential targets of a certain miRNA were predicted by PicTar (Krek *et al*., 2005), TargetScan (Release 6.2: June 2012) (Lewis *et al*., 2003) and the overlaps of results from both programs composed the final predicted targets. The predictions were according to the interactions of human mRNA-miRNA due to the absence of porcine miRNA in current version of above-mentioned algorithm. The Gene Ontology (GO) terms and KEGG pathway terms enriched in predicted target genes were performed using a DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/home.jsp).

Quantitative real-time PCR (qPCR): The expression changes of 9 selected miRNA were validated by an SYBR-based High-Specificity miRNA qRT-PCR Detection kit (TaKaRa, Dalian, China) on the CFX96™ Real-Time PCR Detection System (Bio-Rad, CA, USA). The qPCR validation were carried out on three biological replicates. The endogenous control genes U6 snRNA were used in this assay. The $2^{-\Delta\Delta C}$T Method was used to determine the expression level difference between surveyed samples.

RESULTS AND DISCUSSION

Summary of high-throughput sequencing data: As a result, 13.84 Million (M) and 18.62 M raw reads were generated from immature and mature testes, respectively. Of these, 6.10 M (immature testis) and 13.60 M (mature testis) were deemed as the high-quality reads after filtering out the reads that did not meet the accepted criteria.

The size distribution of high-quality reads was similar in the immature and mature libraries (Pearson's $r = 0.94$, $p<0.001$). In both libraries, the 22-nucleotide (nt) small RNA was the most abundant (immature: 42.21%; mature: 44.16%) followed by 21 and 23 nt small RNAs (Fig. 1) which is consistent with the typical size of miRNAs from Dicer-derived products and agrees with previous deep sequencing results (Burnside *et al*., 2008). Intriguingly in

the mature library, researchers also observed a weak peak at approximately 27-29 nt which may be the testis-specific piwi-interacting RNAs (Klattenhoff and Theurkauf, 2008; Lian *et al*., 2012).

Identification and characterization of the miRNA transcriptome of immature and mature testes: A total of 461 mature miRNAs corresponding to 227 miRNA precursors (pre-miRNAs) were identified in the two libraries. As shown in Table 1, the identified mature miRNAs and their precursors were divided into the following three subgroups in order from high to mid confidence (Ambros *et al*., 2003):

- Porcine known miRNAs (with genome location): 370 miRNAs corresponding to 213 known porcine pre-miRNAs which are also mapped to the pig genome. Specifically, 256 are known in miRBase and 114 were novel miRNAs that were identified in this study
- Porcine known miRNAs (without genome locations): 26 miRNAs mapped to 15 known porcine pre-miRNAs. Specifically, 19 were in miRBase 19.0 and 7 were novel miRNAs
- Porcine conserved miRNAs: 65 miRNAs corresponding to 49 other known mammalian pre-miRNAs in miRBase 19.0 and these pre-miRNAs mapped to the pig genome. miRBase 19.0 (Aug. 2012) deposited 271 pre-miRNAs encoding 306 known porcine miRNAs

In this study, researchers found 259 and 261 known porcine miRNAs in immature and mature testes, respectively. Altogether, there was a very high detection rate (275 of 306, 89.87%) of known porcine miRNAs, illustrating that the immature and mature libraries encompass almost the entire repertoire of previously known miRNAs which are essential for the various biochemical pathways during spermatogenesis.

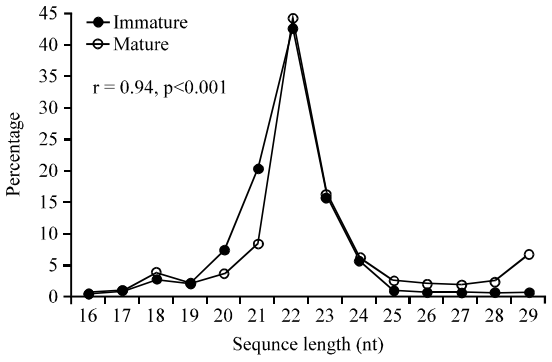

Fig. 1: The length distribution of sequences

miRNAs highly expressed in porcine testes: The identified miRNAs exhibited a large dynamic range of read counts ranging from three to millions. As shown in Fig. 2, researchers found that the top 10 unique miRNAs with the highest abundance contributed 58.88 and 77.52% of the total unique read counts in immature (Fig. 2a) and mature libraries (Fig. 2b), respectively. This result indicated that only a few types of miRNAs comprised the majority of sequences. Intriguingly, five unique miRNAs were shared in the top 10 positions of the two libraries.

The high abundance of these miRNAs that were highly expressed in both immature and mature libraries implies that they may have housekeeping cellular roles and may be the main regulatory miRNAs in spermatogenesis and basic cellular metabolism (Copley *et al*., 2013).

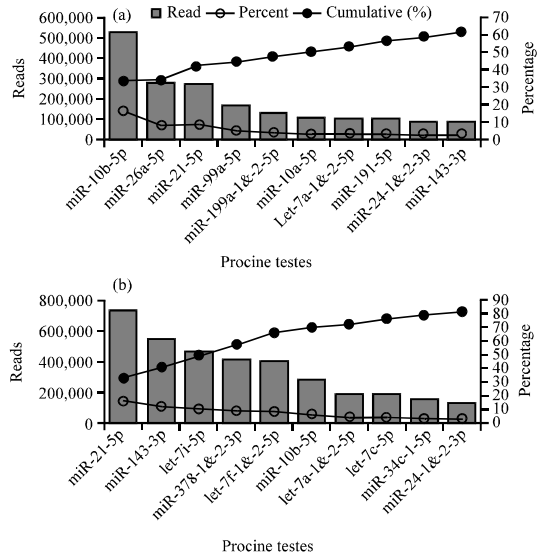

Fig. 2: Top 10 most highly expressed unique miRNAs; a) Top 10 most highly expressed unique miRNAs in immature porcine testes; b) Top 10 most highly expressed unique miRNA in mature porcine testes

Table 1: Pre-miRNAs and mature miRNAs identified in porcine sexually immature and mature testes

Group description	Immature miRNA/pre-miRNA	Mature miRNA/pre-miRNA	Total miRNA/pre-miRNA
Group 1: known miRNAs (with genome loctation)	335/200	339/205	370/213
Group 2: known miRNAs (without genome location)	25/15	23/13	26/15
Group 3: conserved miRNAs	44/33	52/40	65/49
Total	404/248	414/258	461/277

For example, previous reports have shown that the let-7-family of miRNAs is ubiquitously expressed in various cell and tissue types with a high expression level and is involved in the cell cycle as a master regulator of cell proliferation pathways (Roush and Slack, 2008; Barh *et al*., 2010). A recent report showed that miR-21 may contribute to cellular crosstalk in the tumor microenvironment (Nouraee *et al*., 2013). miR-143 plays an important regulatory role in odontoblast differentiation (Liu *et al*., 2013), tumor angiogenesis (He *et al*., 2013a, b) and cancer treatment (Clape *et al*., 2009; Sachdeva and Mo, 2010). The miR-10b is increased in lymphatic metastatic tissues (Chen *et al*., 2013) and high-grade breast tumor vasculature (Plummer *et al*., 2013). The miR-24 is abnormally expressed in asthenozoospermic and oligoasthenozoospermic males compared with normozoospermic males (Abu-Halima *et al*., 2013). In summary, the findings and other evidence support the significant relevance of the most abundant miRNAs to testicular physiology and also suggest that these miRNAs may participate in the regulation of numerous physiological processes and many diseases.

miRNAs differentially expressed between immature and mature porcine testes: As shown in Fig. 3a, 325 of 428 unique miRNAs (75.94%) were co-expressed in immature and mature libraries and 47 (10.98%) and 56 (13.08%) miRNAs appear to be preferentially expressed in immature and mature libraries, respectively. In total, researchers identified 303 unique miRNAs (out of 428, 70.79%) with statistically significant differential expression between immature and mature testes. Out of these 303 DE unique miRNAs, 208 (immature specific: 18, co-expressed: 190) and 95 (mature specific: 17, co-expressed: 78) unique miRNAs are down and up-regulated in immature versus mature testis, respectively.

To validate the high-throughput sequencing data, nine DE miRNAs (i.e., ssc-miR-202-5p, ssc-miR-10b-5p, ssc-miR-99a-5p,ssc-miR-26a-5p,ssc-miR-204-5p,ssc-miR-146a-5p, ssc-miR-142-5p, ssc-miR-155-3p and ssc-miR-10a-3p) were selected randomly to quantify their relative expression levels using quantitative Polymerase Chain Reaction (qPCR). As shown in Fig. 3b, the results showed that there was a significant positive correlation between the high-throughput sequencing and qPCR results (Pearson's r = 0.96, $p<10^{-3}$) which highlighted the high reliability of the sequencing data.

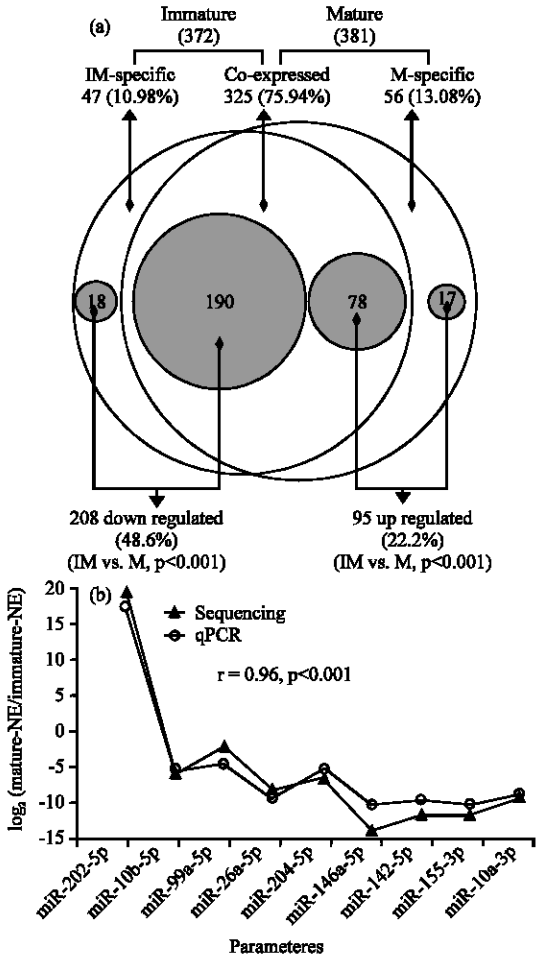

Fig. 3: Characteristics of the Differentially Expressed (DE) unique miRNAs; a) The Venn diagram displays the distribution of 428 unique miRNAs between immature testis (left circle) and mature testis (right circle) libraries. The gray circles indicate the DE unique miRNAs ($p<0.001$, Bonferroni corrected) in immature versus mature testes; b) Quantitative Polymerase Chain Reaction (qPCR) validation for the nine DE miRNAs. Pearson's correlation was used to determine the relationship between the qPCR and high-throughput sequencing data for miRNA expression levels. Mature-NE and Immature-NE represent normalized expression levels for the miRNAs in the mature and immature libraries, respectively

miRNA target gene prediction and functional annotation: As previously reported, the more abundantly miRNAs were regarded as being more important than the less abundantly miRNAs and they exerted their regulatory function in many pathways (Carlsbecker *et al*., 2010; Miyashima *et al*., 2011; Ma *et al*., 2013). Eventually, 20 highly abundant DE miRNAs were selected to predict target genes and perform Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (The top 10 miRNAs that were the most enriched

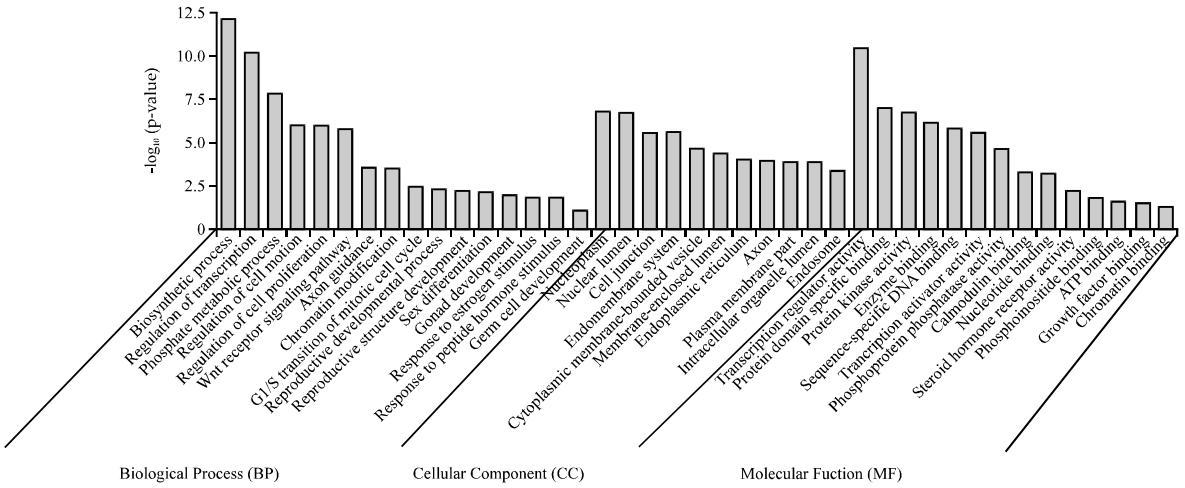

Fig. 4: Partial Gene Ontology (GO) classification annotated for predicted target genes

in the matureand immature libraries, respectively ($p<0.001$, reads >1000)). In total, researchers identified 3118 target genes and the GO annotations indicated that these target genes were mainly enriched in regulation of biosynthesis process, signal transduction and energy metabolism such as DNA transcription, phosphate metabolic process, chromatin binding and response to steroid hormone stimulus (Fig. 4). The KEGG pathway analysis identified 54 enriched pathways for these highly abundant DE miRNAs and the pathways in cancer, MAPK signaling pathway, focal adhesion, Wnt signaling pathway, neurotrophin signaling pathway and GnRH signaling pathway ranked top among the enriched pathways (Table 2).

As reported by resent studies, MAPK signaling pathway (Almog and Naor, 2008; Das *et al*., 2013; Zhang *et al*., 2013a, b), Wnt signaling pathway (Das *et al*., 2013) and GnRH signaling pathway were known to be involved in the regulation of spermatogenesis and spermatozoa function and in controlling embryonic development in organisms. This study further highlighted the significant roles of these abundant DE miRNAs during spermatogenesis.

Chromosomal mapping of miRNAs: The porcine X chromosome is 144.29 Mbp in size ranking as the 10th largest among all of the pig chromosomes. Intriguingly, in this study, 33 pre-miRNAs (11.91%, 33 of 277), 55 miRNAs (11.93%, 55 of 461) and 43 DE miRNAs (12.87%, 43 of 334) were located on the X chromosome (Fig. 5), respectively and all of these indexes ranked first in the X chromosome among all pig chromosomes (except the Y chromosome). Consistent with previous reports, researchers further found a higher density (2.14 fold) of pre-miRNA loci (Guo *et al*., 2009; Li *et al*., 2010, 2011), higher

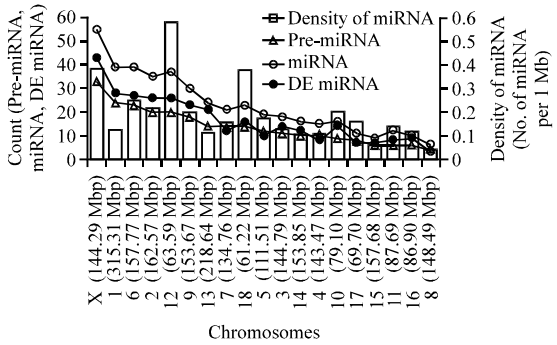

Fig. 5: Chromosomal mapping of porcine testes miRNAs

Table 2: Partial enriched KEGG pathways of target genes for the most abundant DE miRNAs

Pathways	Genes count	p-values
Pathways in cancer	104	9.70×10^{-13}
MAPK signaling pathway	86	5.27×10^{-11}
Neurotrophin signaling pathway	51	6.46×10^{-11}
Wnt signaling pathway	52	5.51×10^{-8}
Colorectal cancer	35	7.07×10^{-8}
GnRH signaling pathway	35	4.82×10^{-6}
Axon guidance	42	6.59×10^{-6}
ErbB signaling pathway	32	6.75×10^{-6}
Focal adhesion	56	2.88×10^{-5}
p53 signaling pathway	25	8.78×10^{-5}
Oocyte meiosis	31	1.99×10^{-3}
Insulin signaling pathway	36	2.27×10^{-3}
Cell cycle	33	4.25×10^{-3}
Calcium signaling pathway	38	5.09×10^{-2}

density (2.13 fold) of miRNAs (Zhang *et al*., 2013a, b) and higher density (2.35 fold) of DE miRNAs on the X chromosome (0.23 pre-miRNA loci per Mb of chromosome, 0.38 miRNAs per Mb of chromosome and 0.30 DE miRNAs per Mb of chromosome, respectively) than the average of 18 other autosomes (0.11 pre-miRNA loci per Mb of chromosome, 0.18 miRNAs per Mb of

chromosome and 0.13 DE miRNAs per Mb of chromosome, respectively). These results suggested that both miRNAs and DE miRNAs that were transcribed from porcine testes were preferentially located on the X chromosome.

CONCLUSION

This study effectively extended the repertoire of porcine testis miRNAs and compared the miRNA transcriptomes between sexually immature and mature porcine testes. In addition, researchers identified many DE miRNAs and found that the majority of miRNAs and DE miRNAs originated from the X chromosome. Furthermore, GO and KEGG pathway analyses of the predicted target genes suggested the important roles for these DE miRNAs in spermatogenesis. In summary, the study indicates that miRNAs are extensively involved in spermatogenesis and that unraveling miRNA functions in the testis will further the understanding of the regulatory mechanisms of mammalian spermatogenesis and male infertility treatment.

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