Profiling of miRNAs in porcine germ cells during spermatogenesis

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Abstract

Spermatogenesis includes mitosis of spermatogonia, meiosis of pachytene spermatocytes and spermiogenesis of round spermatids. MiRNAs as a ~22 nt small noncoding RNA are involved in regulating spermatogenesis at post-transcriptional level. However, the dynamic miRNAs expression in the developmental porcine male germ cells remains largely undefined. In this study, we purified porcine spermatogonia, pachytene spermatocytes and round spermatids using a STA-PUT apparatus. A small RNA deep sequencing and analysis were conducted to establish a miRNAs profiling in these male germ cells. We found that 19 miRNAs were differentially expressed between spermatogonia and pachytene spermatocytes, and 74 miRNAs differentially expressed between pachytene spermatocytes and round spermatids. Furthermore, 91 miRNAs were upregulated, while 108 miRNAs were downregulated in spermatozoa. We demonstrated that *ssc-miR-10a-5p*, *ssc-miR-125b*, *ssc-let-7f* and *ssc-miR-186* were highly expressed in spermatogonia, pachytene spermatocytes, round spermatids and spermatozoa respectively. The findings could provide novel insights into roles of miRNAs in regulation of porcine spermatogenesis.

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Introduction

Spermatogenesis is a complex process occurring in the epithelium of seminiferous tubules within testis (Aravin & Bourc'his 2008). Spermatogonial stem cells (SSCs), a subpopulation of undifferentiated spermatogonia, are the foundation of spermatogenesis (Kanatsu-Shinohara & Shinohara 2013). Spermatogonia enter into meiotic process to become spermatocytes, which undergo a long-lasting meiosis and transform into haploid round spermatids (Miller *et al.* 2013). Subsequently, round spermatids accomplish spermiogenesis. Eventually spermatozoa are released into the lumen of seminiferous tubules and matured during transit along epididymis (Guyonnet *et al.* 2011, Rathke *et al.* 2014).

This highly organized process of spermatogenesis requires timely coordinated gene expression that is regulated in transcriptional and post-transcriptional level (Yao *et al.* 2015). Growing evidences have revealed that a large number small noncoding RNAs are involved in the regulation of spermatogenesis (Kimmins & Sassone-Corsi 2005, Papaioannou & Nef 2010, Chen *et al.* 2017b). miRNAs, as 22 nt noncoding RNA, regulate gene

expression by inducing mRNA degradation or inhibiting translation (Martinez & Tuschl 2004). Increasing evidences suggest that miRNAs play important roles in regulating SSCs self-renewal and differentiation (Niu *et al.* 2011, He *et al.* 2013, Moritoki *et al.* 2014), meiosis (Yu *et al.* 2005, Novotny *et al.* 2007), spermiogenesis (Korhonen *et al.* 2011) and sperm functions (Liu *et al.* 2012) in mice. The dynamically expressed miRNAs of spermatogonia and primary spermatocytes have been analyzed in mice (Smorag *et al.* 2012). Importantly, miRNA signatures of human spermatogonia, pachytene spermatocytes and round spermatids were unveiled recently, which provided new targets for the treatment of male infertility (Liu *et al.* 2015).

It has been estimated that as little as 40% of research data obtained from rodents yield successful human clinical trials (Vandamme 2015). In terms of anatomy, physiology and function, swine is considered close to humans. As such, swine is steadily gaining importance as large animal models in the field of biomedical research (Swindle *et al.* 2012). In previous study, Luo and coworkers detected the miRNAs profiling in immature and mature porcine testis tissues (Luo *et al.* 2010). The

authors further analyzed miRNA expressions in porcine testes, epididymis and ejaculated spermatozoa (Luo *et al.* 2015). Moreover, recently Chen and coworkers identified differentially expressed miRNAs in epididymal and ejaculated spermatozoa (Chen *et al.* 2017a). However, the expression pattern of miRNAs in porcine male germ cells remains largely unknown.

The aim of the present study was to isolate the different types of porcine male germ cells and to establish a comprehensive miRNAs profiling in these isolated cells via a small RNA deep sequencing and analysis. This study would provide novel insights into roles of miRNAs in the regulation of porcine spermatogenesis.

Materials and methods

Animal

All experimental procedures involving animals were approved by the Northwest A&F University's Institutional Animal Care and Use Committee. The testes of puberty Landrace (5-month-old) were obtained from Besun farm, Yangling, China. After the castration, testes were put into pre-cooled PBS (4°C) contained 2% of penicillin and streptomycin. The samples were brought to the lab immediately.

Sample collection

The isolation of porcine germ cells was described in previous study (Zheng *et al.* 2014). Briefly, the testis was cut into small pieces and incubated in collagenase Type IV (0.2% w/v; Invitrogen) at 37°C for 30 min to obtained seminiferous tubules. The seminiferous tubules were digested by 0.25% trypsin-EDTA (Hyclone, Logan, UT, USA) at 37°C for 15 min. After filtration, the single cell was collected by centrifugation and subjected to differential plating to remove the adherent cells.

The STA-PUT apparatus via the velocity sedimentation was utilized to enrich spermatogonia (A), pachytene spermatocytes (PS) and round spermatids (RS) from cell suspension. The process was strictly carried out as described by Liu *et al.* 2015. The different cell types were collected and rigorously examined under a phase-contrast microscope to identify cellular morphology and cell types. The cells of each type were pooled and re-suspended in TRIzol (Invitrogen) and stored at -80°C until usage. The samples from each type of germ cells were prepared for immunocytochemistry and qRT-PCR analysis.

The fresh ejaculated spermatozoa (SP) were collected from healthy adult Landrace pig using the gloved hand technique. The semen was incubated in pre-warmed water (37°C) and delivered to laboratory immediately. The upper fraction of swim-up spermatozoa was collected followed by centrifuging at 600g for 5 min. After the removing of supernatant, the sperm pellets were re-suspended in TRIzol and stored at -80°C.

All samples were collected from two individuals and considered as two biological replicates.

Quantitative RT-PCR (qRT-PCR)

RNA was extracted from the isolated spermatogonia, pachytene spermatocytes, round spermatids, spermatozoa with TRIzol (Invitrogen) according to the manufacturer's protocol. For each sample, total RNA was used for reverse transcription using PrimeScript RT reagent kit with gDNA Eraser (Takara). The quality of the resultant cDNAs was verified by PCR analysis of β -actin expression. The primer information is shown in the Supplementary Table 1 (see section on supplementary data given at the end of this article). qRT-PCR was performed with SYBR Green II PCR Mix (Takara) using an IQ-5 (Bio-Rad). The relative gene expression level was normalized to β -actin and analyzed using the $2^{-\Delta\Delta CT}$ method. For real-time PCR of miRNA, stem-looped primers (Supplementary Table 2) were used for reverse transcription as described previously (Tang *et al.* 2006). The relative expression level of miRNAs was normalized to 5s RNA and calculated using the comparative Ct method ($2^{-\Delta\Delta CT}$).

Immunocytochemistry and meiotic spread assay

To identify the cells, the freshly isolated spermatogonia, round spermatids were fixed with 0.4% paraformaldehyde (PFA) for 30 min at room temperature and washed with cold PBS for three times. The cells were further permeabilized for 5 min using 0.4% Triton-X 100 (Sigma-Aldrich) followed by washing with PBS. After blocked nonspecific reaction in 1% BSA for 30 min at room temperature, the cells were incubated with primary antibodies, including GPR125 (G protein-coupled receptor 125, Abcam), GFRA1 (GDNF family receptor A1, Abcam), UCHL1 (ubiquitin C-terminal hydrolase L1, AbD Serotec, Germany), SCP3 (synaptonemal complex protein 3, Abcam), Acrosin (Santa Cruz, USA) and PRM2 (Protamine 2, Santa Cruz, USA) at a dilution with 1:200 overnight at 4°C. Next day, the cells were washed with PBS for three times and incubated with rhodamine-conjugated secondary antibody IgG (Sigma-Aldrich) at a dilution with 1:200 for 1 h at room temperature. For labeling nucleus, cells were washed and counterstained with DAPI (CWBIO, Beijing, China). A fluorescence microscope (Leica) was used for fluorescence observation and photographing.

For identification of the isolated pachytene spermatocytes, meiotic spread assays were conducted strictly based on previous study (Liu *et al.* 2015). Briefly, a hypotonic solution was used for preparing cell lysis which was spread evenly over slides layered with 1% PFA and 0.15% Triton-X-100. After drying for 24 h at room temperature in a humid chamber, the cells on the slides were treated with 0.04% photoflo for 5 min and blocked with 4% normal serum. The cells were incubated with primary antibodies including SCP3 (Abcam) and CREST (nBAF chromatin remodeling complex subunit, Immunovision, USA) overnight at 37°C in a humid chamber. The secondary antibody Alexa 555 donkey anti-rabbit (Molecular Probes) and Alexa 350 goat anti-mouse (Molecular Probes) were used to incubate for 90 min at 37°C. After washing with TBS, the cells were treated with antifade

and fluorescence images were taken by a fluorescence microscope (Leica).

RNAseq, data processing and analysis

Small RNA deep sequencing was carried out by Shanghai Institute of Biochemistry and Cell Biology, which provided an improved deep sequencing method (Yang *et al.* 2016). In brief, cDNA libraries of small RNAs were constructed using 10 ng of total RNA. After the adaptor ligation, first-strand cDNA synthesis and PCR amplification, the 130 bp–160 bp DNA fragments corresponding to the small RNA fraction were acquired. Subsequently, an Illumina HiSeq 2000 sequencer was used for the sequencing of small RNA libraries. For data analysis, small RNA sequence adaptors from the Illumina sequencing were first removed and other contaminants were filtering out using software developed by BGI. Then, read sequences longer than 18 nt were used for mapping to the libraries of miRNAs, tRNAs, rRNAs, sn/snoRNAs (small nuclear/small nucleolar RNA) and repeat elements. The small RNA tags were mapped to reference sequence (Sus_scrofa.Sscrofa10.2) by Bowtie to analyze their expression and distribution on the reference. Mapped small RNA tags were used to look for known miRNAs. miRBase was used as reference, modified software mirdeep2 was used to obtain the potential known miRNAs. The percentage and length distribution of small RNAs was based on the acquired read counts. The differential expressed miRNAs between spermatogenesis stages were analyzed with R DEGseq package. Then, acquired miRNA expression level was normalized by the equation (read counts $\times$ 1,000,000)/library size (total miRNA read counts) and presented as TPM. And the volcano, PCA and heatmap were all drew by R according to the differential expressed miRNAs TPM. miRNA targets were predicted by miRanda. Alignment score of 160 or greater and an energy threshold of -10.0 kcal/mol or less targets were considered as candidates. And alignment score of 170 or greater and an energy threshold of -20.0 kcal/mol

or less targets were used for functional annotation and further verification.

Luciferase reporter assay

Reverse transcription of RNA from porcine testis tissue was performed according to manufacturer's instruction of Transcriptor First-Strand cDNA synthesis kit (Roche). Gene 3' UTR was amplified and cloned into the dual-luciferase reporter vector psiCHECK2 at the site digested with NotI and XhoI. Primers are listed in the [Supplementary Table 1](#).

psiCHECK2 with targeted gene 3'UTR were cotransfected with miRNA mimics/NC into Hela cells using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturing protocols. After 48 h, all target validation assays were performed with the Dual-Luciferase Assay System (Promega). Firefly luciferase activity was normalized to renilla luciferase activity.

Statistical analysis

SPSS, V17.0 statistical software was used for statistical analysis. All values were presented as mean $\pm$ S.E.M., and statistically significant was consider when P value less than 0.05(*), 0.01(**).

Results

Identification of male germ cells

Porcine male germ cells at different stages during spermatogenesis were isolated by STA-PUT apparatus via the velocity sedimentation. The cell types were verified under a phase-contrast microscope based on the cell morphological and phenotypic characteristics. The diameters of spermatogonia, pachytene spermatocytes and round spermatids were around 10, 15 and 5 μ m, respectively (Fig. 1A). Immunocytochemistry analysis further revealed that the isolated spermatogonia were positive for GPR125, GFRA1 and UCHL1 (Fig. 1B),

Figure 1 Identification of porcine male germ cells. (A) The morphological characteristics of freshly isolated porcine spermatogonia (A), pachytene spermatocytes (PS) and round spermatids (RS) under a phase-contrast microscope. (B) Immunocytochemistry revealed the expression of GFRA1, GPR125 and UCHL1 in spermatogonia. Meiosis spread assays displayed the expression of SCP3 and CREST in the freshly isolated pachytene spermatocytes. Acrosin and PRM2 were identified to express in the round spermatids. (C) Percentage of positive staining cell was determined. qRT-PCR was conducted to determine the specific marker genes for male germ cells (*SOHLH1* for spermatogonia, *SCP3* for pachytene spermatocytes and *PRM1* for spermatids). Data are presented as the mean $\pm$ S.E.M., ** $P < 0.01$. Scale bars in A and B = 10 μ m.

pachytene spermatocytes positive for SCP3 and round spermatids positive for Acrosin and PRM2. Meiosis spread assay indicated that SCP3 and CREST were co-expressed in the isolated pachytene spermatocytes. Moreover, the purity of isolated germ cells was more than 88% (Fig. 1C). To further verify the purity of the isolated cells, expression of marker genes (*SOHLH1* for spermatogonia, *SCP3* for pachytene spermatocytes, and *PRM1* for round spermatids) was detected (Gan et al. 2011). Analysis showed that *SOHLH1* (spermatogenesis and oogenesis-specific basic helix-loop-helix 1), *SCP3* and *PRM1* (protamine 1) were significant highly expressed in the isolated spermatogonia, pachytene spermatocytes and round spermatids, respectively (Fig. 1C). Taken together, these results suggest that spermatogonia, pachytene spermatocytes and round spermatids were successfully isolated from the mixed germ cells.

Profiling of small RNAs

In total, 7 samples were successfully sequenced with the Illumina Hiseq 2000 sequencer and used for the study. Total acquired reads varied from 8.8 million to 14.2 million. After adaptor sequences were trimmed, the quality reads were mapped to the porcine genome. And the mapped ratio of filter reads were 95.4, 95.4, 95.1, 93.9, 94.2, 83.3 and 83.9% for samples of spermatogonia (A), pachytene spermatocytes 1 (PS1), pachytene spermatocytes 2 (PS2), round spermatids 1 (RS1), round spermatids 2 (RS2), spermatozoa 1 (SP1), spermatozoa 2 (SP2), respectively (Table 1). The double peak of small RNA was detected in all cell types, whose peaks were distribution at the length of 20–25 nt and ~30 nt. The first peak around 20–25 nt was lower than the second peak located on ~30 nt in spermatogonia, pachytene spermatocytes and round spermatids (Fig. 2B, C, D, E and F). Conversely, the first peak of spermatozoa was higher than the second peak in spermatozoa (Fig. 2G and H). As shown in Fig. 2A, miRNAs were highly abundant in spermatozoa, which can also be reflected by the distribution of small RNA categories according to the length (Fig. 2G and H). In addition, the high peak of small RNA around 30 nt means the expression of PIWI-interacting RNAs (piRNAs) in spermatogonia, pachytene spermatocytes and round spermatids. These

observations indicate the dynamic expression level of small RNAs in spermatogenesis.

Dynamic expression of miRNA during porcine spermatogenesis

In order to identify the differential expressed miRNA between contiguous stages, R DEGseq package was used for calculating. Scatter plot was used to show the differentially expressed miRNA between continually stages during spermatogenesis (Fig. 3A, B and C, Supplementary Table 3). Six upregulated miRNAs and 13 downregulated miRNAs were identified between pachytene spermatocytes and spermatogonia (Table 2). In addition, 70 miRNAs were upregulated and 4 miRNAs were downregulated in round spermatids compared with pachytene spermatocytes (Table 2). Finally, 199 miRNAs were differentially expressed between spermatozoa and round spermatids, reflecting that these differences may in part derive from epididymal secretion during spermatozoa transit from caput to caudal region (Table 2). Functional annotation showed that miRNAs differentially expressed between pachytene spermatocytes and spermatogonia may be involved in cell differentiation, development, cell signal transduction (Supplementary Fig. 1A). The target genes between round spermatids and pachytene spermatocytes were involved in cell localization, migration and response to stress (Supplementary Fig. 1B). Furthermore, target gene clusters of differentially expressed miRNAs between spermatozoa and round spermatids participate in regulation of metabolic process, development process and programmed cell death (Supplementary Fig. 1C). Principal component analysis (PCA) suggested that pachytene spermatocytes were similar to round spermatids (Fig. 3D). Meanwhile, miRNAs expression in spermatogonia and spermatozoa was different with that in pachytene spermatocytes and round spermatids (Fig. 3D). The minor differences between pachytene spermatocytes biological replicates or round spermatids biological replicates were due to the differences of sample derived individual animals.

Differentially expressed miRNAs between continual stages during spermatogenesis were considered as dynamically expressed miRNAs and used for hierarchical clustering analysis (Supplementary Fig. 2). In order

Table 1 Data processing metrics for small RNA reads for specific development stages.

Sample	Total reads	Quality reads	Reads for mapping	Mapped reads	Mapped ratio
A	14,025,211	13,505,759	13,186,169	12,573,691	95.40%
PS1	14,794,199	14,207,264	13,879,331	13,241,856	95.40%
PS2	11,051,084	10,603,406	10,370,530	9,865,042	95.10%
RS1	13,936,919	13,327,024	12,841,061	12,055,271	93.90%
RS2	10,763,730	10,345,374	10,089,406	9,507,414	94.20%
SP1	11,936,011	11,411,295	10,845,902	9,029,783	83.30%
SP2	8,880,017	8,476,409	8,007,996	6,717,602	83.90%

A, Spermatogonia; PS, Pachytene Spermatocytes; RS, Round Spermatids; SP, Sperm.

Figure 2 Categories and length distribution of small RNAs. (A) Percentage of different categories of small RNAs mapped to the porcine genome in each developmental stage. (B, C, D, E, F, G and H) Composition of small RNA categories according to length distribution in each sample. Spermatogonia (A), pachytene spermatocytes (PS), round spermatids (RS), spermatozoa (SP).

to further verify miRNAs expression in specific germ cells, miRNAs with high read counts and differentially expressed among different stages were chosen for further determination. Analysis revealed that the expression of *ssc-miR-10a-5p* in spermatogonia was significantly higher than that in pachytene spermatocytes, round spermatids, but was similar to that in spermatozoa (Fig. 4A). Furthermore, the expression of *ssc-miR-125b* in pachytene spermatocytes was significantly higher than that in round spermatids and spermatozoa, but comparable with that in spermatogonia (Fig. 4B). In addition, *ssc-let-7f* and *ssc-miR-186* exhibited the highest abundance in round spermatids and spermatozoa, respectively (Fig. 4C and D). Together, these results further confirmed the quality and authenticity of the RNA sequencing data obtained in this study.

Prediction and verification of miRNA targets

According to the miRanda prediction, alignment score more than 170 and energy threshold less than -20kcal/mol candidates were selected for target verification. As shown in Fig. 5, *MAP2K6* (mitogen-activated protein kinase kinase 6), *LRRC8A* (leucine-rich repeat-containing 8 family member A), *CDC6* (cell division cycle 6) and *CDC42* (cell division cycle 42) were considered as candidate targets of *ssc-miR-10a-5p*, *ssc-miR-125b*, *ssc-let-7f* and *ssc-miR-186* respectively. To identify the function of miRNAs, genes 3'UTR was cloned into psi-check2 downstream from the renilla luciferase coding region. Each reporter construct was separately co-transfected with the miRNA mimics or negative control RNA into Hela cells. For *ssc-miR-10a-5p*, the luciferase activity was downregulated 19.2% by transfecting with *ssc-miR-10a-5p* mimics compared to negative control RNA (Fig. 6A). Similarly, the declined percentage of luciferase activity for *ssc-miR-125b*, *ssc-let-7f* and

ssc-miR-186 were 33.2, 50 and 63.5% respectively (Fig. 6B, C and D). These results demonstrate that *MAP2K6*, *LRRC8A*, *CDC6* and *CDC42* were the target of *ssc-miR-10a-5p*, *ssc-miR-125b*, *ssc-let-7f* and *ssc-miR-186* respectively.

Discussion

Fluorescent-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS), based on cell surface markers, are most frequently used methods for cell purification. Several markers have been identified for isolating gonocytes or undifferentiated spermatogonia (Tokuda *et al.* 2007, Zheng *et al.* 2009, Aloisio *et al.* 2014). Although CD90 (Ryu *et al.* 2004, Zheng *et al.* 2014), $\alpha 6$ -integrin and $\beta 1$ -integrin (Shinohara *et al.* 1999) have been proven as useful surface markers for

Figure 3 The relationship among different phases according to differentially expressed genes and PCA. (A) Scatter plot comparison revealed the patterns of genes between A and PS. (B) Scatter plot comparison revealed the patterns of genes between PS and RS. (C) Scatter plot comparison revealed the patterns of genes between RS and SP. Standard selection criteria to identify differentially expressed genes was established at P value < 0.01 ; the $\log_2$ Fold change > 0.5 were expressed as up genes (red dots), $\log_2$ Fold change < -0.5 were expressed as down genes (green dots). (D) Two main principal components explain the correlation among A, PS, RS, SP.

Table 2 The number of differentially expressed miRNAs.

Item	Comparison	Upregulated	Downregulated
1	PS vs A	6	13
2	RS vs PS	70	4
3	SP vs RS	91	108

Figure 4 Verification of the expression level of miRNAs at each developmental stage. (A) The expression level of *ssc-miR-10a-5p* during spermatogenesis. (B) The expression level of *ssc-miR-125b* during spermatogenesis. (C) The expression level of *ssc-let-7f* during spermatogenesis. (D) The expression level of *ssc-miR-186* during spermatogenesis.

isolating male germ stem cells, lacking of appropriate surface markers hindered the application of FACS and MACS for isolation of pachytene spermatocytes and round spermatids. Alternatively, STA-PUT has been successfully used for purification of spermatogonia, pachytene spermatocytes and round spermatids in mice and humans (Gan et al. 2011, Liang et al. 2014, Liu et al. 2015). However, it has not been applied to male germ cells in livestock animals. In this study, we applied STA-PUT to isolate porcine male germ cells. The isolated germ cells displayed normal morphology with a proper diameter and were positive for specific cell markers, indicating that spermatogonia, pachytene spermatocytes and round spermatids were successfully isolated in pigs. This study would provide as a reference for the isolation of male germ cells in other livestock species.

MiRNAs are small non-coding RNA that plays important roles in regulating spermatogenesis (McIver et al. 2012, Kotaja 2014, Yao et al. 2015). Analysis of miRNA transcriptome indicated that 224 unique

miRNAs were differentially expressed between porcine ovary and testis. Meanwhile, miRNA located on the X chromosome exhibited a testis-preferential or testis-specific pattern (Li et al. 2011). In addition, there were 51 upregulated miRNAs and 78 downregulated miRNAs when comparing immature and mature testis (Luo et al. 2010). Recently, the miRNAs expression pattern in testis, epididymis and ejaculated spermatozoa were revealed. The co-expressed miRNAs were involved in the cell cycle process, metal ion binding, plasma membrane modification and p53 signal pathway (Luo et al. 2015). Furthermore, the variety of small RNAs in pig epididymal spermatozoa, ejaculated spermatozoa and seminal plasma was also analyzed (Chen et al. 2017a). In order to further understand the miRNAs expression pattern during porcine spermatogenesis, we conducted a small RNA deep sequencing of porcine-specific stages germ cells during spermatogenesis. Meanwhile, we systematically analyzed the characteristics of miRNAs expression. The analysis revealed that miRNAs were differentially expressed among porcine spermatogonia, pachytene spermatocytes, round spermatids and spermatozoa. In addition, 212 miRNAs were differentially expressed in the different types of male germ cells, suggesting that these miRNAs might be involved in regulating porcine spermatogenesis. Recent reports revealed the expression pattern of miRNAs during mouse (Smorag et al. 2012) or human spermatogenesis (Liu et al. 2015). Through profiling microarray data, Smorag and coworkers identified 45 miRNAs that were dynamically expressed in mouse SSCs, premeiotic cells and primary spermatocytes (Smorag et al. 2012). Similarly, Liu and coworkers found that 56 miRNAs were differentially expressed in human spermatogonia, pachytene spermatocytes and round spermatids (Liu et al. 2015). In the present study, using a small RNA deep sequencing analysis, we identified 212 miRNAs that were dynamically expressed in porcine spermatogonia, pachytene spermatocytes, round spermatids and spermatozoa (Supplementary Fig. 2). Particularly, several miRNAs (*miR-125b*, *-10a*, *-99a*, *-99b*) in porcine spermatogonia and spermatocytes exhibited a similar expression pattern as in human and mouse (Smorag et al. 2012, Liu et al. 2015), suggesting that

Figure 5 Predicted targets and binding sites of differentially expressed miRNAs. (A) The predicted binding sites of *ssc-miR-10a-5p* in *MAP2K6* 3'UTR. (B) The predicted binding sites of *ssc-miR-125b* in *LRRC8A* 3'UTR. (C) The predicted binding sites of *ssc-let-7f* in *CDC6* 3'UTR. (D) The predicted binding sites of *ssc-miR-186* in *CDC42* 3'UTR.

Figure 6 Targets validation of differentially expressed miRNAs using dual-luciferase assay. (A) *MAP2K6* was a target of *ssc-miR-10a-5p*. (B) *LRR8A* was a target of *ssc-miR-125b*. (C) *CDC6* was a target of *ssc-let-7f*. (D) *CDC42* was a target of *ssc-miR-186*. Data shown are the mean $\pm$ S.E.M., ** $P < 0.01$.

these miRNAs may play a conserved roles in regulating spermatogonial proliferation and meiotic initiation. Conversely, *let7* family miRNA (*let-7a*, *7c*, *7f*) was highly expressed in porcine pachytene spermatocytes and round spermatids, whereas this family was exceedingly expressed in human and mouse spermatogonia (Smorag et al. 2012, Liu et al. 2015). In addition, *miR-34c* was differentially expressed in porcine spermatozoa. In previous studies, *miR-34c* was detectable in pig ejaculated spermatozoa, epididymal spermatozoa and seminal plasma (Chen et al. 2017a). However, it was highly expressed in human spermatogonia (Liu et al. 2015), mouse SSCs (Niu et al. 2011), mouse pachytene spermatocytes and round spermatids (Smorag et al. 2012). Moreover, *miR-34b/c* and *miR-449a/b/c* knockout mouse model showed *miR-34c* played important roles in spermatogenesis (Yuan et al. 2015). These findings suggested that miRNAs play conserved and significant role in spermatogenesis.

Previous study has shown that a few miRNAs play vital roles in regulating SSC self-renewal and differentiation (Gou et al. 2014). *miR-10a* has been demonstrated to regulate differentiation (Foley et al. 2011, Okamoto et al. 2012), to modulate cell senescence (Zhu et al. 2013), to inhibit cell proliferation and promote cell apoptosis (Fan et al. 2016). Additionally, *miR-10a* is involved in retinoic acid induced the sequential programs of spermatogonial differentiation and

the entry in meiosis (Huang et al. 2010, Meseguer et al. 2011, Busada & Geyer 2016). However, *miR-10a* functions in regulating spermatogenesis remain largely unclear. In the present study, we found that *ssc-miR-10a-5p* was highly expressed in porcine spermatogonia and spermatozoa, and that *MAP2K6* was a target of *ssc-miR-10a-5p*. The function of *ssc-miR-10a-5p* in regulating spermatogonial proliferation and differentiation in pigs is worthy of study.

Evidence showed that *miR-125b* was significantly upregulated with testicular RA (retinoic acid) intervention when dogs were administrated with CYP26B1 (cytochrome P450 family 26 subfamily B member 1) inhibitor and all-trans-RA (Kasimanickam et al. 2014). In line with previous findings in human and mouse (Smorag et al. 2012, Liu et al. 2015), we observed that *ssc-miR-125b* was highly expressed in porcine spermatogonia and pachytene spermatocytes. We also identified that *ssc-miR-125b* can target *LRR8A*, which has been demonstrated to activate AKT pathway and played essential roles in development, survival of T cells (Kumar et al. 2014). Meanwhile, our unpublished data have revealed that PI3K/AKT pathway was involved in regulation of survival of spermatogonial stem cells. Taken together, we speculate that *ssc-miR-125b*- *LRR8A*-AKT acts as a candidate pathway in regulating survival of porcine spermatogonia and pachytene spermatocytes.

Additionally, we demonstrated that *ssc-let-7f* was highly expressed in round spermatids and targeted 3'UTR of *CDC6*. Previous study revealed that *CDC6* was essential for meiotic maturation in oocytes (Lemaitre et al. 2002, Eward et al. 2004) and to avoid unscheduled DNA replication during meiotic maturation (Daldello et al. 2015). Comparably, the upregulation of *ssc-let-7f* may participate in the accomplishment of spermatocytes meiosis by regulating *CDC6* expression.

Remarkably, *ssc-miR-186* can be used as a reference for qRT-PCR in spermatozoa because of its constant expression level (Zhang et al. 2015). In the present study, we found that *ssc-miR-186* was presented with high abundance in porcine spermatozoa and could target *CDC42*. Interestingly, *CDC42* has been demonstrated to be expressed higher in asthenozoospermic group spermatozoa than normospermic samples (Salvolini et al. 2013). In addition, *CDC42* could arrest capacitation and subsequently inhibit the acrosome reaction (Baltierrez-Hoyos et al. 2012). Therefore, it is worth to elucidate whether *ssc-miR-186* regulates capacitation and acrosome reaction via *CDC42* in pigs.

Lastly, round spermatids undergo spermiogenesis including acrosomal biogenesis, flagellum development, chromatin condensation, cytoplasmic reorganization and exclusion, and eventually transform into spermatozoa, which are released into the lumen of seminiferous tubules and further

become mature in epididymis (Rathke *et al.* 2014). During the maturation in epididymis, spermatozoa plasma membrane is modified by exosomes that are secreted from epididymal epithelia (Nixon *et al.* 2015). The exosomes derived from the ampulla of ductus deferens and sexual glands (seminal vesicles, prostate and bulbourethral glands) further decorate the plasma membranes (Aalberts *et al.* 2014) during ejaculation. The miRNAs could be delivered to spermatozoa mediated with the exosomes during sperm maturation and ejaculation process. Therefore, it is not surprising that only 36.81% transcripts were common between bovine spermatids and mature spermatozoa (Gilbert *et al.* 2007). Recently, Chen and coworkers conducted a comparative analysis of small RNA profiles in porcine ejaculated spermatozoa, epididymal spermatozoa and seminal plasma (Chen *et al.* 2017a). The authors found that over 70% of small RNAs detected from ejaculated spermatozoa are the ejaculated spermatozoa specific. Compared with epididymal spermatozoa, 117 miRNAs were downregulated, 26 were upregulated and 102 were unchanged in ejaculated spermatozoa (Chen *et al.* 2017a). These upregulated sperm-borne small RNAs may originate from the exosomes. Importantly, the lack of comparison between porcine round spermatids and ejaculated spermatozoa was replenished in the present study. We found that 7 of the top 10 highly expressed miRNAs across the three libraries reported previously by Chen and coworkers (Chen *et al.* 2017a) were differentially expressed between round spermatids and spermatozoa. In the current study, spermatogonia, pachytene spermatocytes and round spermatids were collected from the same individual, and ejaculated spermatozoa was collected from another individual. Whether a proportion of the observed variance between spermatozoa and the other germ cells are the resulting from individual difference deserves further validation.

In summary, we successfully isolated porcine spermatogonia, pachytene spermatocytes and round spermatids from adult testis tissues. Meanwhile, we systematically analyzed the miRNAs in different male germ cells. The findings would provide a new insight into regulation of porcine spermatogenesis.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-17-0441>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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