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CD49f-Positive Testicular Cells in Saanen Dairy Goat Were Identified as Spermatogonia-Like Cells by miRNA Profiling Analysis

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ABSTRACT

miRNAs, a type of small RNA, play critical roles in mammalian spermatogenesis. Spermatogonia are the foundation of spermatogenesis and are valuable for the study of spermatogenesis. However, the expression profiling of the miRNAs in spermatogonia of dairy goats remains unclear. CD49f has been one of the surface markers used for spermatogonia enrichment by magnetic activated cell sorting (MACS). Therefore, we used a CD49f microbead antibody to purify CD49f-positive and -negative cells of dairy goat testicular cells by MACS and then analysed the miRNA expression in these cells in depth using Illumina sequencing technology. The results of miRNA expression profiling in purified CD49f-positive and -negative testicular cells showed that 933 miRNAs were upregulated in CD49f-positive cells and 916 miRNAs were upregulated in CD49f-negative cells with a twofold increase, respectively; several miRNAs and marker genes specific for spermatogonial stem cells (SSCs) in testis had a higher expression level in CD49f-positive testicular cells, including miR-221, miR-23a, miR-29b, miR-24, miR-29a, miR-199b, miR-199a, miR-27a, and miR-21 and CD90, Gfra1, and Plzf. The bioinformatics analysis of differently expressed miRNAs indicated that the target genes of these miRNAs in CD49f-positive cells were involved in cell-cycle biological processes and the cell-cycle KEGG pathway. In conclusion, our comparative miRNAome data provide useful miRNA profiling data of dairy goat spermatogonia cells and suggest that CD49f could be used to enrich dairy goat spermatogonia-like cells, including SSCs. J. Cell. Biochem. 115: 1712–1723, 2014. © 2014 Wiley Periodicals, Inc.

KEY WORDS: miRNAs; CD49f; TESTICULAR CELLS; MAGNETIC ACTIVATED CELL SORTING; SPERMATOGONIAL STEM CELLS; DAIRY GOAT; ILLUMINA SEQUENCING

miRNAs are important regulators of gene expression that mainly play critical functions post-transcriptionally to control the expression of target mRNAs. Spermatogenesis is governed by orchestrated, phase-specific gene expression patterns that are tightly controlled at the transcriptional and post-transcriptional levels. Increasing evidence has shown that several classes of miRNAs are expressed in male germ cells and play essential roles in small-RNA-mediated regulation of spermato-

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genesis [Yadav and Kotaja, 2014]. miRNAs promoting cell cycling were highly expressed in primordial germ cells (PGCs) and spermatogonia, and they are important for the proliferation and early differentiation of spermatogonia in spermatogenesis [Hayashi et al., 2008].

Spermatogonia, including spermatogonial stem cells (SSCs), are progenitor cells that maintain spermatogenesis, produce spermatozoa and transmit genetic information from the parents to the descendants [Brinster, 2002, 2007]. The isolation, purification and cultivation of spermatogonia are the foundation for real-time follow-up studies of spermatogenesis in vitro. However, there have still been heterogeneous cells such as Sertoli cells and myoid cells in the spermatogonia purified by traditional methods (enzymatic digestion and differential plating), making improved methods necessary. Magnetic activated cell sorting (MACS) is capable of obtaining high vitality of cells during the sorting process and does not affect the subsequent culture of the isolated cells. Several cell purification studies using MACS combined with surface markers of spermatogonia have been reported, and many spermatogonial markers have been identified in humans, rodents and other domestic animals. CD49f is one of the surface markers identified for the enrichment of mouse and bovine spermatogonia by MACS [Shinohara et al., 1999; de Barros et al., 2012]. Our previous study found that CD49f was also localised in dairy goat spermatogonia and that the expression levels of CD49f, Plzf, Oct4, Gfra1, Vasa were much higher in Thy1-positive goat spermatogonia [Wu et al., 2013], suggesting CD49f as a candidate marker for dairy goat spermatogonia cells.

The current Illumina high-throughput sequencing technology is highly efficient and sensitive for the detection of miRNA expression. An earlier report had shown that specific miRNAs are temporally and spatially differentially expressed in spermatogonial cells [Smorag et al., 2012]. The spermatogonial cells are of particular utility in animal genetics, breeding and reproduction, provided that gene transfection and homologous transplantation of spermatogonia are combined to produce transgenic animals with improved productivity and commercial value [Zheng et al., 2014].

Dairy goats, as economically important animals, have a relatively short period of gestation and provide milk, meat, fur and other valuable products. Dairy goats are also used as animal models for biomedical research in the production of peptide medicines by transgenic animals [Ko et al., 2000]. However, the miRNA profiling in purified spermatogonia of goat has not been investigated. Due to the lack of putative miRNA expression profiles and spermatogonial markers, it is necessary to purify spermatogonial cells from whole testicular cells to understand the mechanism of goat spermatogenic meiosis [Abu-Halima et al., 2014] and establish dairy goat SSCs for further biotechnological studies. Therefore, we purified CD49f-positive and negative testicular cells by MACS and analysed the miRNA expression in depth with Illumina high-throughput sequencing technology. The results showed that, among the identified novel and known miRNAs, 933 and 916 miRNAs were upregulated with a twofold increase in CD49f-positive and CD49f-negative testicular cells, respectively. The enriched miRNAs and genes putatively assigned to spermatogonia had a higher level expression in dairy goat CD49f-positive testicular cells and included miR-221, miR-23a, miR-29b, miR-24, miR-29a, miR-199b, miR-199a, miR-27a, miR-21 and Gfra1, Plzf. GO and pathway analysis of the potential miRNA target genes indicated that the enriched miRNAs are involved in important functions in dairy goat testis, including cell-cycle progression. This study provides some newly useful miRNA profiling data for dairy goat spermatogonia cells and demonstrates a useful method for enriching the spermatogonia-like cells from dairy goat testis.

MATERIALS AND METHODS

ISOLATION OF DAIRY GOAT TESTICULAR CELLS

Three testes from three 5-month-old dairy goats were collected and delivered to the laboratory on ice. Then, the testicular cells were separated in our laboratory as previously reported [Zhu et al., 2012], using CDD mixed digestive enzyme: 2 mg/ml collagenase (Invitrogen, Carlsbad, CA) + 20 μg/ml DNase (Invitrogen) + 2 mg/ml decomposition enzyme (Invitrogen).

ENRICHMENT OF CD49f-POSITIVE DAIRY GOAT TESTICULAR CELLS

Differential plating was performed to remove potential contamination of Sertoli cells and myoid cells. The testicular cells were placed in a 10-cm-diameter culture dish in DMEM/F12 medium supplemented with 10% FBS (Hyclone, Logan, UT) at 37°C overnight. Subsequently, the non-attached cells were collected for MACS by centrifuging at 1,000 rpm for 5 min.

The CD49f-positive testicular cells were obtained by MACS using a MiniMACS separation unit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), as in our previous report [Wu et al., 2013]. The testicular cells (approximately 1.3×10^7 cells) were resuspended in 80 μl MACS buffer (PBS containing 2 mM EDTA and 0.5% BSA) and incubated with anti-CD49f (ITGA6) monoclonal antibody for 1 h at 37°C, followed by incubation with 20 μl anti-mouse IgG microbead-conjugated antibody for 15 min at 4°C.

SMALL RNA LIBRARY CONSTRUCTION AND DEEP SEQUENCING

The total RNAs of CD49f positive and negative testicular cells were extracted with RNAiso plus reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions, and then the purity and concentration of the total RNAs were detected with the RNA Bioanalyser (Agilent Technologies, CA).

A small RNA library was generated from the two RNA samples with the Illumina Truseq Small RNA Preparation kit, according to Illumina's TruSeq Small RNA Sample Preparation Guide. The purified cDNA library was used for cluster generation on Illumina's Cluster Station and then sequenced on Illumina GAIIx, following the vendor's instructions. Raw sequencing reads were obtained with Illumina's Sequencing Control Studio software, version 2.8 (SCS v2.8), followed by real-time sequencing image analysis and base-calling by Illumina's Real-Time Analysis version 1.8.70 (RTA v1.8.70). The extracted sequencing reads were stored as files and then used in the sequencing data analysis.

SEQUENCING DATA ANALYSIS

The sequencing data were analysed with the ACGT101-miR v4.2 package (LC Sciences, Houston, TX), as previously reported [Li et al., 2010; Wu et al., 2014]. Matching sequences met the following the classification of generated sequence groups: A, mapped to selected pre-miRNAs in miRbase v19.0 (Groups 1, 2 and 3); B, unmapped to selected pre-miRNAs in miRbase v19.0, but mapped to the goat genome (Group 4); C, mapped to another defined database (Groups 5 and 6). The details are shown in Table S1.

TARGET GENE PREDICTION AND BIOINFORMATICS ANALYSIS OF DIFFERENTIALLY EXPRESSED miRNAs

Because a 3′-UTR database is currently unavailable for predicting the putative target genes of differentially expressed miRNAs, we first built a goat 3′-UTR database by searching in the 3UTRef Database (http://www.ba.itb.cnr.it/BIG/Blast/BlastUTR.html) with the goat mRNA database in NCBI (http://www.ncbi.nlm.nih.gov/nuccore/?term=(txid9925)±AND±%22mrna%22%5BFilter%5D). Then, to predict the target genes of miRNAs, the RNAhybrid software program [Kruger and Rehmsmeier, 2006] was used to predict targets. To comprehensively describe the properties of genes and gene products, the cow genes corresponding to the target goat genes of differentially expressed miRNAs in CD49f-positive and negative testicular cells were analysed with the DAVID Bioinformatics Resources 6.7 Database [Huang et al., 2009]. The GO terms were significantly enriched for the predicted target gene candidates of the miRNAs when compared with the reference gene background and the genes corresponding to biological functions. Comparison with the reference gene background identified KEGG pathways significantly enriched for the candidate target genes. The GO terms and enriched KEGG pathways with $P \leq 0.05$ were considered credible.

QUANTITATIVE RT-PCR ANALYSIS

The total RNAs from CD49f-positive and CD49f-negative dairy goat testicular cells were reverse transcribed to cDNAs using the M-MuLV Reverse Transcriptase reagent kit according to the reagent manual (Thermo Scientific, FL). Real-time quantitative PCR was performed on a CFX96 real-time PCR detection system (Bio-Rad, CA) according to the manual for the BioEasy SYBR Green I Real-Time PCR kit (Bioer Co. Ltd, Hangzhou, China). Relative expression levels of target genes and differentially expressed miRNAs were normalised to Gapdh and 5S RNA expression for each sample, respectively; the relative expression levels were calculated using $2^{-\Delta\Delta Ct}$. The primers for the validated mRNAs and miRNAs are listed in Table S2a,b.

FACS ANALYSIS

For FACS-mediated cell-cycle analysis, the dairy goat CD49f-positive and CD49f-negative testicular cells were washed twice with PBS, and then 10^6 cells were resuspended in 1 ml of 75% alcoholic solution at 4°C overnight. Next day, the pretreated cells were washed three times with PBS before incubation in 500 μl propidium iodide staining medium (10 mg/L propidium iodide and 1 mg/L RNase A in PBS) for 30 min at 37°C in the dark. The cell-cycle analysis was performed on an EPICS ELITE apparatus (Beckmann-Coulter) using the MultiCycle software (Phoenix Flow Systems, Inc.).

IMMUNOFLUORESCENCE ANALYSIS

Dairy goat testes were fixed in 4% formaldehyde overnight, dehydrated through a series of graded alcohols, and embedded in paraffin at 65°C for 6–8 h. For immunofluorescence, 2-μm sections of testes were dewaxed in xylene and rehydrated through a series of graded alcohols, and the antigen was retrieved by citric acid buffer (pH 6.0) microwave antigen retrieval. After blocking with 1% bovine serum albumin (BSA) for a minimum of 30 min, the sections were incubated with primary antibodies, including CD49f (mouse monoclonal antibody, 1:500, Biolegend, San Diego, CA) and Gfra1 (mouse monoclonal antibody, 1:100, Santa Cruz Biotechnology, CA). The appropriate FITC-conjugated secondary antibodies were used according to the manufacturer's manual (1:500, Chemicon, Temecula, CA). The cell nuclei were stained with Hoechst33342. At the same time, the negative controls were stained with secondary antibodies: goat anti-mouse IgG.

RESULTS

SMALL RNA READS IN CD49f-POSITIVE AND CD49f-NEGATIVE TESTICULAR CELLS

Two small RNA libraries were constructed using the small RNA molecules (less than 30 nt) extracted from total RNA of the CD49f-positive and CD49f-negative testicular cells analysed with the RNA Bioanalyser (Fig. S1). The workflow for the sequencing data analysis is shown in Figure S2. The rRNA, tRNA, snRNA, scRNA, srpRNA and snoRNA sequences were separated out and discarded by using a BLAST tool against the known noncoding RNAs deposited in the Rfam database and NCBI GenBank databases (Fig. S3). Low-quality reads, adapter contaminant sequences and sequencing reads including reads with inserting fragments, reads containing poly(A) stretches and reads of less than 15 nt were removed. Finally, a total of 13,397,862 clean reads representing 6,161,105 unique miRNA sequences were recovered from the CD49f-positive testicular cells library, and 9,310,105 clean reads representing 1,933,750 unique miRNA sequences were recovered from the CD49f-negative testicular cells library (Fig. S3 and Table S3). There were 933 miRNAs with expression at least twofold higher in CD49f-positive cells than in CD49f-negative cells; 1032 miRNAs expressed equally in CD49f-positive and CD49f-negative cells, showing a change between zero- and twofold; and 916 miRNAs with expression at least twofold higher in CD49f-negative cells than in CD49f-positive cells. The unique miRNAs were divided into three groups: high-count miRNAs (counts $\geq$ 7,445), middle-count miRNAs ($10 \leq$ counts $<$ 7,445), and low-count miRNAs ($0 \leq$ counts $<$ 10)(see Table S3). Among the high-count miRNAs, there were some reported miRNAs, including let-7, miR-21, miR-20, miR-146, miR-449, and miR-34c, that were preferentially expressed in spermatogonia and regulated SSC self-renewal or spermatogenesis [Niu et al., 2011; Tong et al., 2011; Bao et al., 2012; He et al., 2013; Huszar and Payne, 2013; Li et al., 2013]. The length distribution and read number percentage of sequences are presented in Figure 1. It was clear that the length distribution of sequenced miRNAs was concentrated from 20 to 24 nt, which is the most common length for miRNAs. There were also some piRNA-like small RNAs belonging to Group 4b (Unmapped to known miRNAs

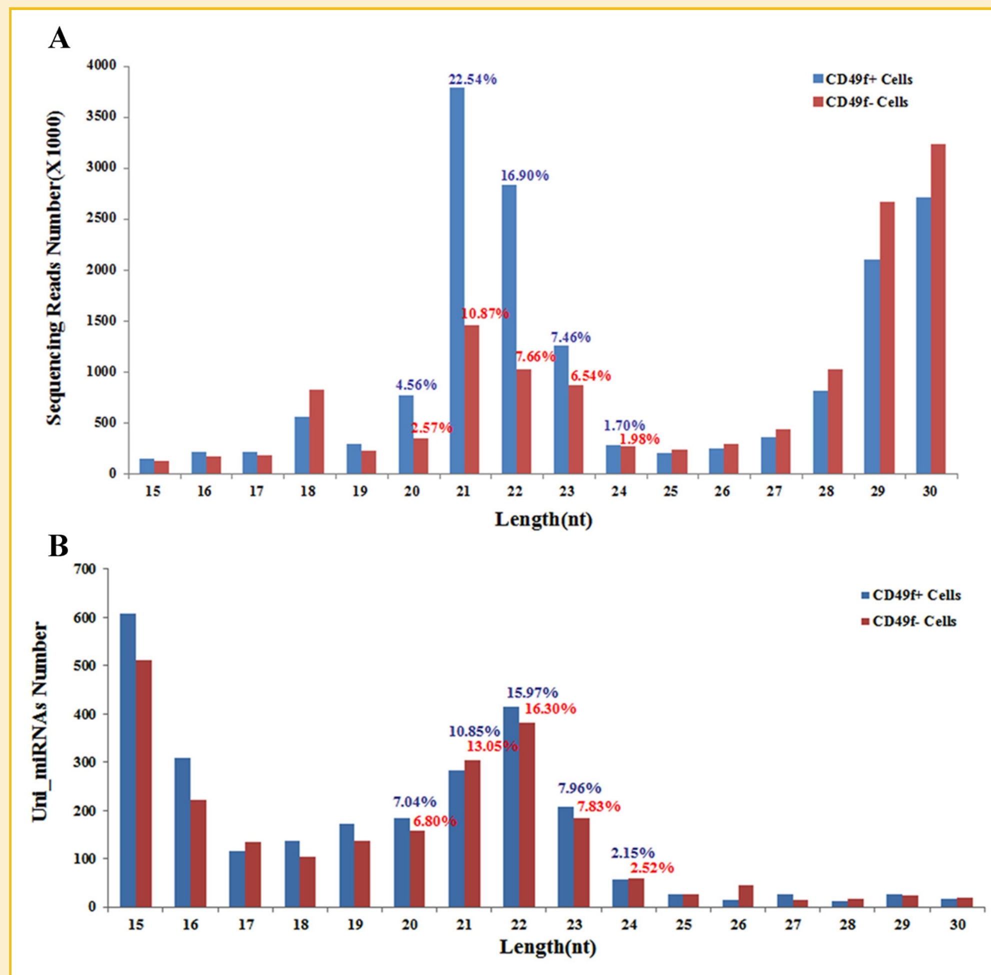

Fig. 1. Length distribution of miRNA sequences in CD49f-positive and CD49f-negative testicular cells. All the reads ranged from 15 to 30 nucleotides in length. A: The length distribution of miRNAs sequences in CD49f-positive and -negative testicular cells based on sequencing reads number, and 53.16% of the miRNA sequences are distributed in the 20–24 nt range in CD49f-positive testicular cells. B: The length distribution of miRNAs sequences in CD49f-positive and -negative testicular cells based on unique miRNA number, and 43.98% of the miRNA sequences are distributed in the 20–24 nt range in CD49f-positive testicular cells. nt, nucleotides; Uni_miRNA, unique miRNA.

but mapped to the *Capra hircus* genome and without hairpins) (see Table S1) and concentrated from 26 to 30 nt as shown in Figure 1. Because the piRNAs have no clear secondary structure motifs and show a bias for a 5′uridine, the piRNA-like small RNA sequences in Group 4b require further analysis and are not further discussed in this present work.

CHROMOSOMAL MAPPING OF GOAT miRNAs

The goat genome database includes the base sequences of 29 pairs of autosomes and the X chromosome [Dong et al., 2013]. Mapping the *Capra hircus* pre-miRNAs from this study to all of the goat chromosomes revealed that miRNAs of CD49f-positive and CD49f-negative testicular cells were evenly distributed on the autosomes and X chromosome by unique miRNA numbers, with more than 10% of miRNAs of CD49f-positive and negative testicular cells mapped to the 21 and X chromosomes (Fig. 2A) by the number of miRNA loci on each chromosome. In contrast, the distribution of miRNAs from libraries for CD49f-positive and CD49f-negative testicular cells was not similar by total unique miRNA copy numbers on each chromosome, with more than 50% of miRNAs mapped to chromosomes 1 and 15 (Fig. 2B).

Post-transcriptional mRNA regulation is active during spermatogenesis, and miRNA-mediated mechanisms are clearly involved in the orchestrated and stage-specific control of gene expression during the entire course of male germ cell differentiation [Yadav and Kotaja, 2014]. Intriguingly, densities of miRNA genes are significantly higher on the mammalian X chromosome than on autosomes as a consequence of the organisation of X-chromosomal miRNA genes in clusters that consist of paralogous copies resulting from miRNA gene duplication [Martin and Seandel, 2013]. Sequencing of spermatocyte and spermatid RNA in comparison to somatic cells revealed that the multimember miRNA families on the X chromosome have higher expression levels than all other miRNA categories in male germ cells [Martin and Seandel, 2013]. In this study, 144 pre-miRNAs from CD49f-positive testicular cells and 121 pre-miRNAs from CD49f-negative testicular cells were located on the X

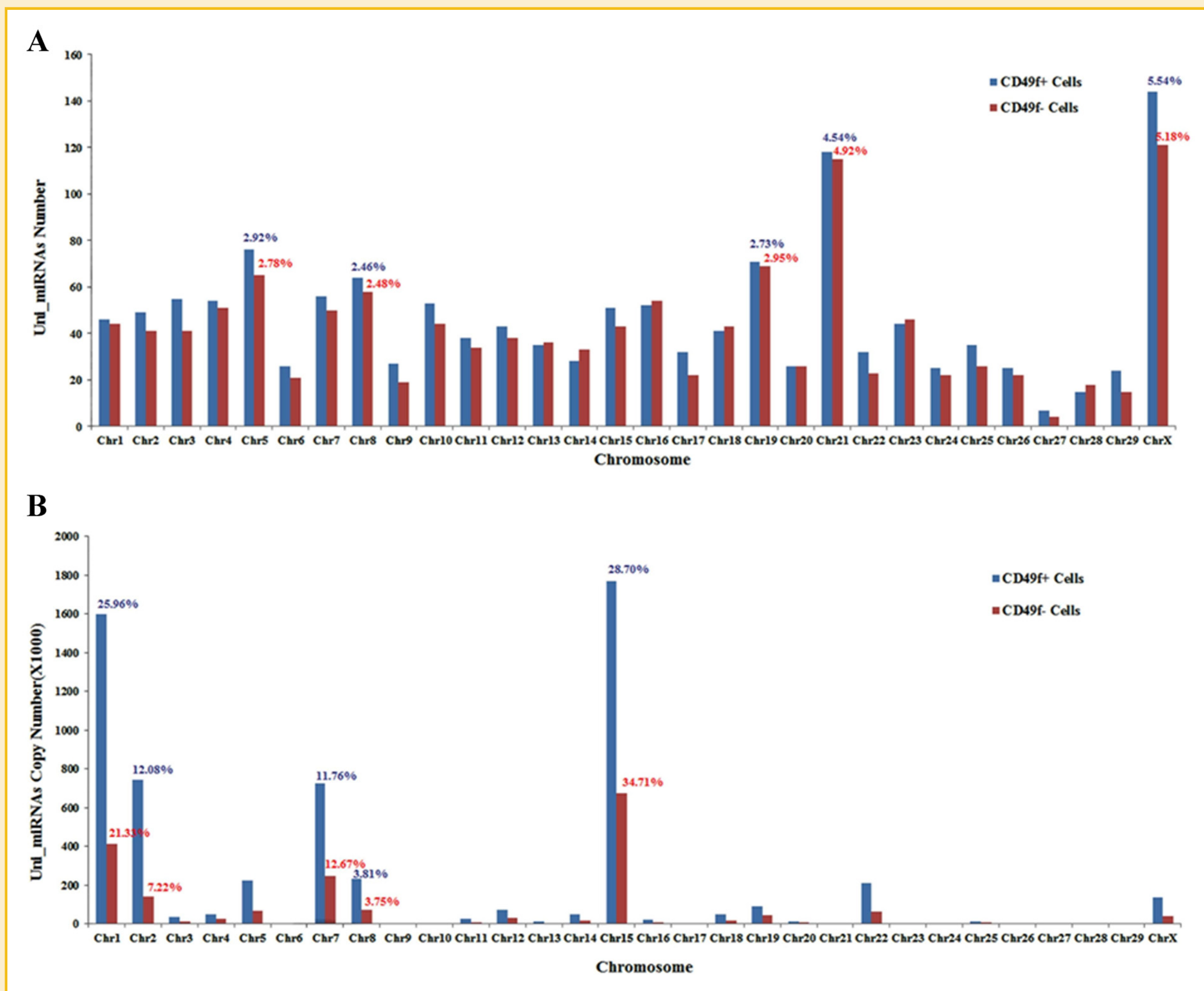

Fig. 2. Chromosomal distribution of miRNAs sequences in CD49f-positive and negative testicular cells. A: The chromosomal distribution of miRNAs sequences in CD49f-positive and -negative testicular cells based on unique miRNA number, the top 5 chromosomes were Chr X, 21, 5, 19 and 8. B: The chromosomal distribution of miRNAs sequences in CD49f-positive and -negative testicular cells based on the copy numbers of unique miRNA, most of the miRNAs were distributed in the top 5 chromosomes, Chr 15, 1, 2, 7 and 8. nt, nucleotides; Chr, Chromosome; Uni_miRNA, unique miRNA.

chromosome, accounting for 5.54% (144/2,599) and 5.17% (121/2,338), respectively, of the genome mapped pre-miRNAs. The distribution of miRNAs among chromosomes usually showed higher densities of miRNAs on the X chromosome compared to the average densities on autosomes in mammalian species, demonstrating that X chromosome miRNA has a role in resistance to meiotic sex chromosome inactivation [Buchold et al., 2010].

VALIDATION OF THE EXPRESSION OF SEQUENCED miRNAs WITH QRT-PCR

To validate the sequencing results of the Saanen dairy goat testicular cells miRNAs, the same RNA preparations used for the deep sequencing were subjected to qRT-PCR. The expression levels of selected miRNAs were determined, including four miRNAs (miR-1468, miR-126-5p, miR-126-3p and miR-10b) with higher read numbers in CD49f-positive testicular cells and 5 miRNAs (miR-9, PC-3p-16208, miR-34c, PC-5p-627 and miR-449b) with higher read numbers in CD49f-negative cells. The qRT-PCR results are shown in Figure 3. The expression pattern of miRNAs was consistent with the deep sequencing results, confirming that the sequencing data were credible.

SPERMATOGONIA-ENRICHED miRNAs IN CD49F-POSITIVE TESTICULAR CELLS

One of the main characteristics of miRNAs is that they are conserved, and some conserved miRNAs, including miR-21, miR-221, miR-34b/c and miR-449, have been found to be important for mammalian spermatogenesis [Wu et al., 2014]. It was reported that, focusing only on the testicles, several conserved miRNAs were specifically expressed in mouse spermatogonia, including SSC-specific miRNAs (miR-221, miR-23a, miR-29b, miR-24, miR-29a, miR-199b, miR-199a, miR-27a, miR-21) and premeiotic-cell-specific miRNAs (let-7a, let-7e, miR-29c); others were specific for meiotic cells, such as miR-425, miR-18a, miR-15b, miR-34b-5p, miR-34b-3p, miR-375, miR-449a, miR-34c [Smorag et al., 2012]. Among the miRNAs with differential expression between the CD49f-positive and

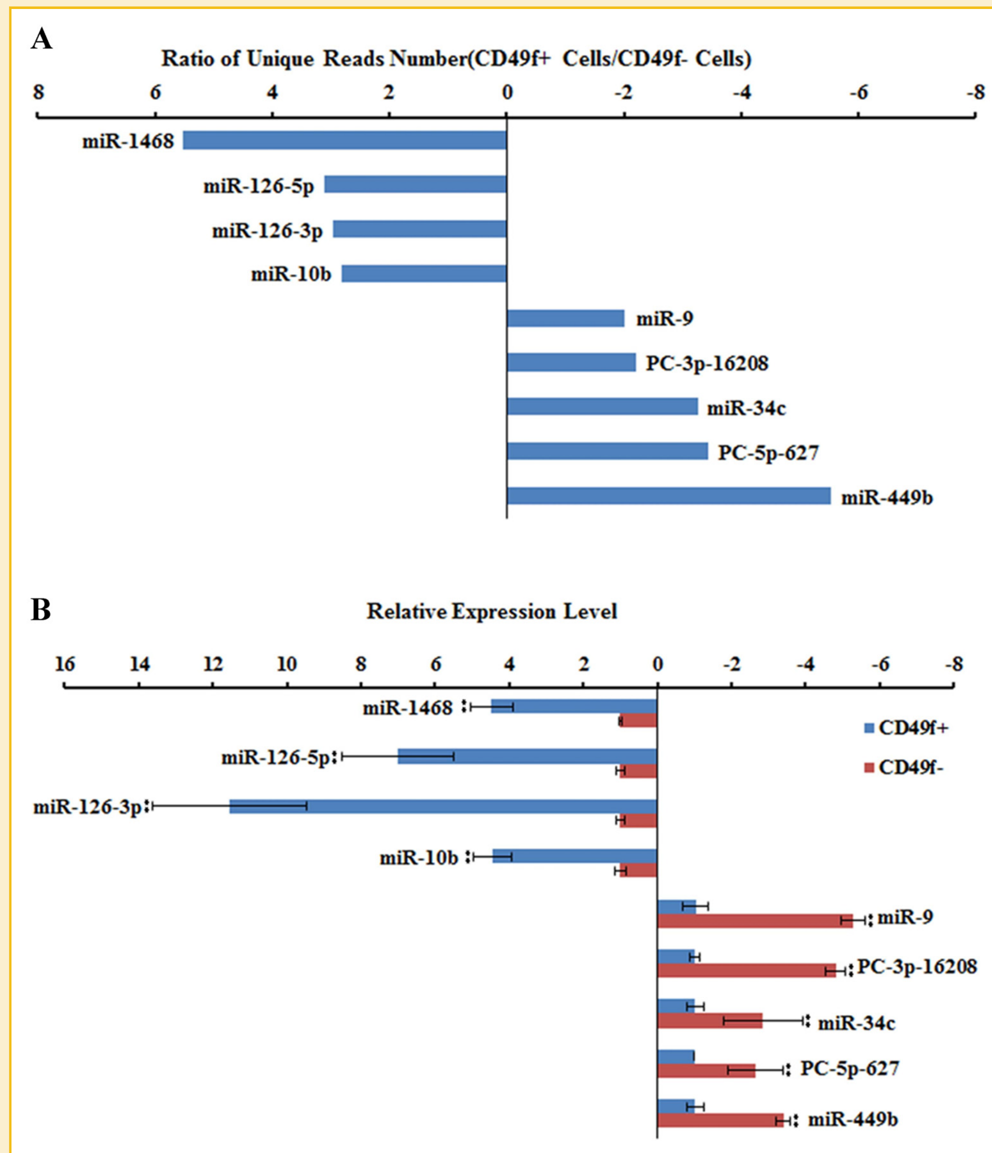

Fig. 3. q-PCR validation of the identified differentially expressed miRNAs by Illumina sequencing. A: The ratio of reads number of some differentially expressed miRNAs (miR-1468, miR-126-5p, miR-126-3p, miR-10b, miR-9, PC-3p-16208, miR-34c, PC-5p-627 and miR-449b) in CD49f-positive and -negative testicular cells by Illumina sequencing. B: The expression levels of miR-1468, miR-126-5p, miR-126-3p, miR-10b, miR-9, PC-3p-16208, miR-34c, PC-5p-627 and miR-449b in CD49f-positive and -negative testicular cells were detected by real-time PCR analysis. 5S RNA was used for normalising the real-time PCR results. The expression patterns of tested miRNAs were consistent with the deep sequencing results. The data are presented as the mean $\pm$ SEM (n = 3). $^{**}P \leq 0.01$.

CD49f-negative testicular cells in dairy goat testis, it was obvious that the miRNAs specific for SSCs and premeiotic cells were expressed more highly in CD49f-positive testicular cells and that the miRNAs specific for meiotic cells were expressed more highly in CD49f-negative testicular cells (Fig. 4). This suggested that the isolated CD49f-positive testicular cells might be a population of dairy goat spermatogonia-like cells.

miRNA TARGET GENE PREDICTION, GO ENRICHMENT AND KEGG PATHWAY ANALYSIS

To further understand the physiological functions and biological processes involving these miRNAs during spermatogonia self-renewal and spermatogenesis, target gene prediction was performed based on miRNA/mRNA interactions to provide some molecular insight into the processes. The target genes and their information were analysed using RNAhybrid software [Kruger and Rehmsmeier, 2006] and DAVID Bioinformatics Resources 6.7 Database [Huang et al., 2009]. A total of 281 and 202 annotated mRNA transcripts were predicted as putative target genes for miRNAs differentially expressed in CD49f-positive and CD49f-negative testicular cells (Table S10).

The GO project provides ontologies to describe attributes of gene products in three non-overlapping domains of molecular biology. Of these, Biological Process (BP) describes biological goals accomplished by one or more ordered assemblies of molecular functions [Harris et al., 2004]. The GO enrichment analysis for biological processes showed that 56 genes of CD49f-positive cells and 100

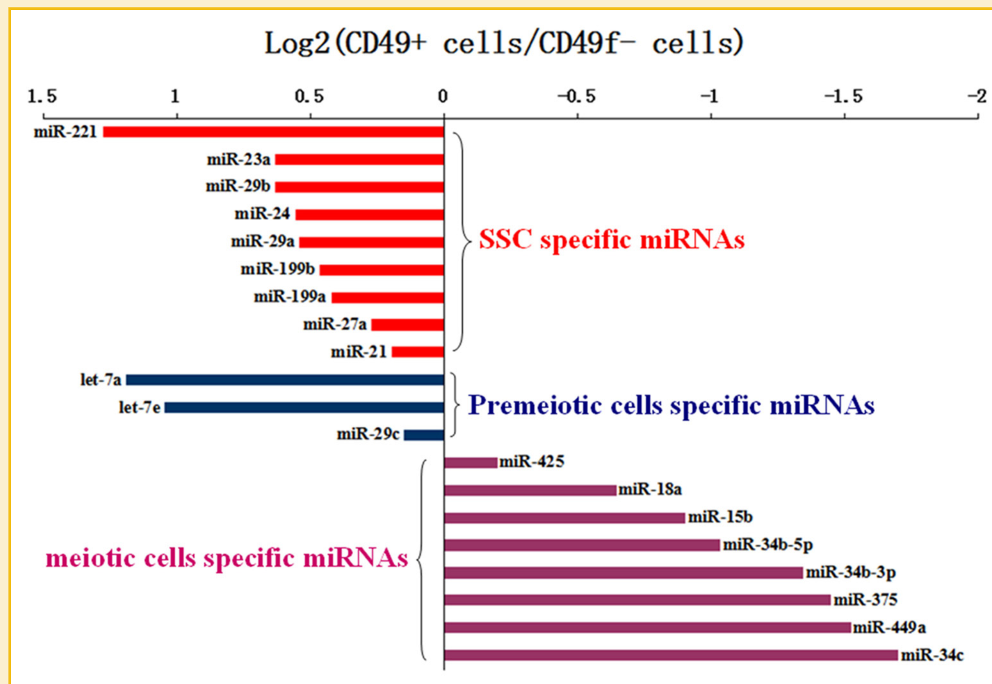

Fig. 4. miRNAs specific to spermatogonial stem cells, premeiotic cells and meiotic cells in CD49f-positive and negative testicular cells by Illumina sequencing. The miRNAs specific to spermatogonial stem and premeiotic cells showed a higher expression level in CD49f-positive testicular cells by miRNAs sequencing. In contrast, the miRNAs specific for meiotic cells were expressed more highly in CD49f-negative testicular cells [Smorag et al., 2012].

genes of CD49f-negative cells were termed from component ontology with a P-value $\leq$0.05. Moreover, 40 genes were involved in transcription, cell cycle and DNA damage processes in CD49f-positive cells (Table I). The KEGG PATHWAY database is supplemented by a set of orthologous group tables for the information about conserved subpathways (pathway motifs), which are especially useful in predicting gene function [Kanehisa and Goto, 2000]. The KEGG Pathway annotation showed only 10 genes of CD49f-positive cells and 7 genes of CD49f-negative cells that were annotated for 1 biological process with $P \leq 0.05$ (Table II). The enriched cell cycle pathway annotated in CD49f-positive cells was consistent with the GO enrichment analysis.

TABLE I. GO Enrichment for the Predicted Target Genes in Biological Processes ($P \leq 0.05$)

CD49f+ Cells			CD49f− Cells		
Gene count	P-Value	Term	Term	P-Value	Gene count
16	4.44E−03	Transcription	Response to wounding	1.19E−03	8
4	6.55E−03	Cell cycle checkpoint	Response to organic substance	1.92E−03	8
3	1.03E−02	Mitotic cell cycle checkpoint	Cell proliferation	3.71E−03	6
3	1.44E−02	DNA damage checkpoint	Defense response	4.68E−03	8
3	1.66E−02	DNA integrity checkpoint	Negative regulation of multicellular organismal process	5.44E−03	5
3	2.15E−02	Determination of left/right symmetry	Smooth muscle contraction	7.28E−03	3
3	2.15E−02	Determination of bilateral symmetry	Oxidation reduction	7.81E−03	12
3	2.15E−02	Determination of symmetry	Feeding behavior	1.33E−02	3
4	3.64E−02	Anatomical structure homeostasis	Inflammatory response	1.60E−02	5
3	3.91E−02	DNA damage response, signal transduction	Regulation of lipase activity	1.76E−02	3
2	4.24E−02	Mitotic cell cycle G2/M transition DNA damage checkpoint	Innate immune response	1.91E−02	4
3	4.59E−02	Peptide transport	Behavior	1.97E−02	6
6	4.94E−02	Cell cycle phase	Regulation of hormone levels	2.00E−02	4
			Regulation of body fluid levels	2.00E−02	4
			Cell–cell signaling	2.49E−02	5
			Peristalsis	2.77E−02	2
			Anion transport	2.95E−02	4
			Behavioral response to ethanol	3.68E−02	2
			Regulation of growth	3.72E−02	5
			Positive regulation of growth	4.42E−02	3

TABLE II. KEGG Pathway for the Predicted Target Genes ($P \leq 0.05$)

CD49f+ Cells			CD49f− Cells		
Gene count	*P*-Value	Term	Term	*P*-Value	Gene count
10	1.04E−05	Cell cycle	Complement and coagulation cascades	1.07E−04	7

THE CELL-CYCLE ANALYSIS OF CD49f-POSITIVE AND NEGATIVE TESTICULAR CELLS

Spermatogonial stem cells were slowly proliferating and self-renewing in G0-G1 transition. FACS-mediated cell-cycle analysis showed that approximately 78% of CD49f-positive testicular cells and 40% of CD49f-negative testicular cells were in G0/G1 phase, and approximately 18% of CD49f-positive testicular cells and 50% of CD49f-negative testicular cells were in G2/M phase (Fig. 5A–C). This suggested that most CD49f-positive testicular cells were in G0/G1 phase and that more than 80% of CD49f-negative testicular cells were in G0/G1 or G2/M phase. Cyclin D1 (CCND1) is an important self-renewal regulator of spermatogonial stem cells and governs the progression of cells from G1 to S-phase [He et al., 2013; Wolgemuth et al., 2013]. Proliferating cell nuclear antigen (PCNA) is a molecular marker that can distinguish various phases in the cell cycle of proliferating spermatogonia, expressed weakly during G1 phase and very intensely during S-G2 phase [Yazawa et al., 2000]. Cyclin A1 (CCNA1) is a cell cycle regulator specific to male germ cells and is involved in the G2-M transition of meiosis I [Liu et al., 1998; Panigrahi et al., 2012]. qRT-PCR showed that the expression level of CCND1 was significantly higher in CD49f-positive than in CD49f-negative testicular cells, and the levels of PCNA and CCNA1 were significantly higher in CD49f-negative than in CD49f-positive testicular cells (Fig. 5D). The FACS and qRT-PCR analyses confirmed the results from the bioinformatics analysis of target genes of differently expressed miRNAs and the

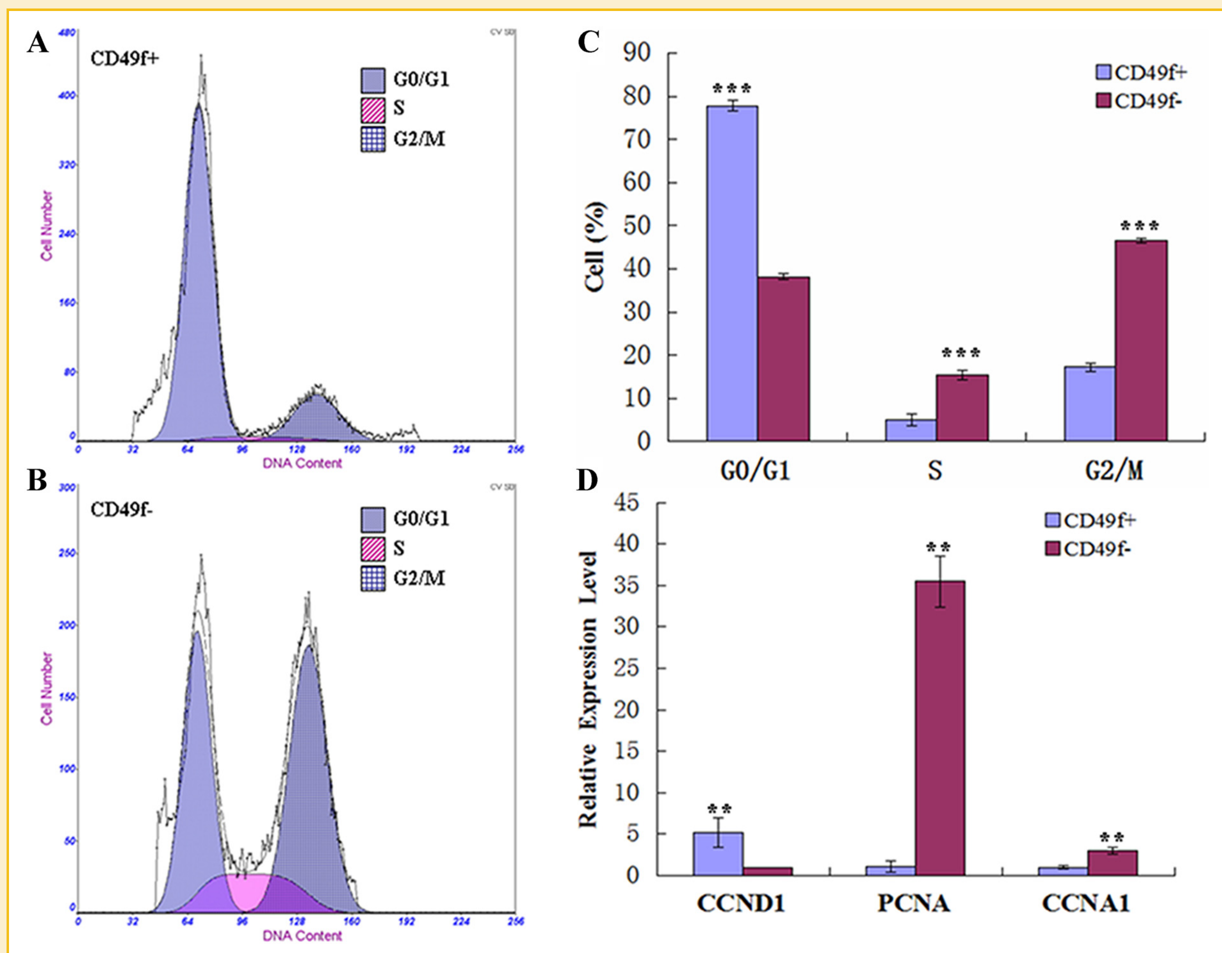

Fig. 5. The cell-cycle analysis of CD49f-positive and CD49f-negative testicular cells. A: The FACS-cell cycle chart of CD49f-positive testicular cells. B: The FACS-cell cycle chart of CD49f-negative testicular cells. C: Statistical analysis of G0/G1, S and G2/M phases in (A,B). D: Different expression levels of CCND1, PCNA and CCNA1 in CD49f-positive and CD49f-negative testicular cells, analysed by quantitative RT-PCR, Gapdh as control for normalization. The data are presented as the mean ± SEM (n = 3). $^{***}P \leq 0.001$; $^{**}P \leq 0.01$.

characteristics of CD49f-positive and CD49f-negative testicular cells.

CD49f-POSITIVE CELLS IN DAIRY GOAT TESTIS

The phenotypic characteristics of dairy goat spermatogonia were explored by using consensus markers for spermatogonia in other species, including CD49f and Gfra1, which was expressed in a subpopulation of spermatogonia [Naughton et al., 2006; He et al., 2007]. An immunofluorescence assay on adult goat testes revealed that Gfra1 was present on the plasma membrane of goat spermatogonia; CD49f was primarily localised in the plasma membrane of goat spermatogonia-like cells lying adjacent to the basement membrane of the seminiferous tubules (Fig. 6A). qRT-PCR analysis showed that CD49f expression increased in testis during the period from 5 to 7 months of age in dairy goats (Fig. 6B). Thus, testes from 5-month-old dairy goats were suitable for isolation of CD49f-positive spermatogonia-like cells.

To characterise the purified goat CD49f-positive spermatogonia-like cells, the expression of CD49f, CD90, Plzf, Gfra1, Oct4, Vasa and Kit was investigated by qRT-PCR. As shown in Figure 6C, except for the differentiated spermatogonia marker Kit [Ohta et al., 2000], all other spermatogonia marker genes (including CD49f [de Barros et al., 2012; Zhu et al., 2012; Sa et al., 2013], CD90 [Reding et al., 2010; Wu et al., 2014], Plzf [Song et al., 2013], Oct4 [Pesce et al., 1998; Sa et al., 2013], Gfra1 [Naughton et al., 2006; He et al., 2007] and Vasa [Zeeman et al., 2002]) were higher in MACS-sorted CD49f-positive cells than in CD49f-negative cells. These results suggested that MACS could help enrich the goat CD49f-positive spermatogonia-like cells.

DISCUSSION

Specific markers of spermatogonia are a useful tool for the isolation and investigation of testicular germ cells. Studies in rodents and primates showed that CD49f was localised to the plasma membrane of the spermatogonia, making it a putative marker for enrichment of spermatogonia from testicular cells [Shinohara et al., 1999; Conrad

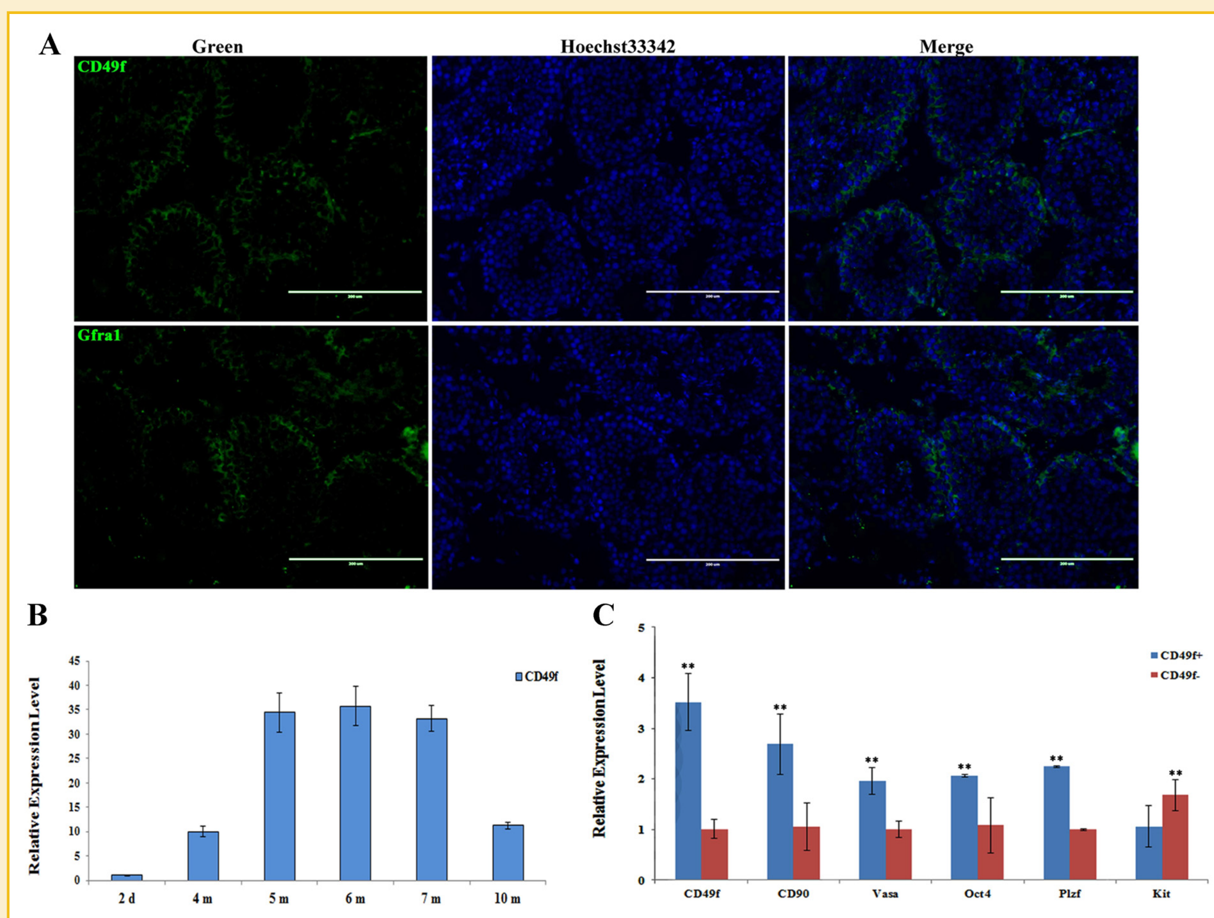

Fig. 6. CD49f-positive cells in goat testis. A: Spermatogonia marker genes in goat testis. Immunofluorescence reveals the expression of Gfra1 and CD49f in goat testes. Gfra1 was present on the plasma membrane of a subpopulation of goat spermatogonia, and CD49f was expressed in similar cells. Scale bars = 100 μm. B: The expression level of CD49f in testis from 2 dpp to 10 months in dairy goat. C: Different expression levels of spermatogonia marker genes in CD49f-positive and CD49f-negative testicular cells. The expression levels of CD49f, Gfra1, Plzf, Oct4 and Vasa were higher in CD49f-positive cells than in CD49f-negative cells, whereas Kit showed the opposite pattern, as analysed by quantitative RT-PCR, Gapdh as control for normalization. The data are presented as the mean ± SEM (n = 3). $^{**}P \leq 0.01$.

et al., 2008; de Barros et al., 2012]. It was also reported that CD49f, CD90, VASA, PLZF, OCT4 and GFRA1 were markers of spermatogonia [Costoya et al., 2004; Naughton et al., 2006; He et al., 2010; Reding et al., 2010; de Barros et al., 2012; Abbasi et al., 2013; Lee et al., 2013; Sa et al., 2013; Song et al., 2013; Wu et al., 2013]. In this study, the isolated and purified dairy goat CD49f-positive testicular cells also expressed these putative markers by immunofluorescence and RT-PCR analysis. Thus, as in other animals, CD49f might be an effective marker for enriching spermatogonia from dairy goats, and the CD49f-positive cell fraction was also a population of spermatogonia in dairy goat.

Illumina high-throughput sequencing has special advantages for sequencing small RNAs and is a powerful tool for the identification of miRNAs. In our study, we found 6,161,105 unique miRNA sequences from the CD49f-positive testicular cells, 1,933,750 unique miRNA sequences expressed in the CD49f-negative testicular cells and 933 miRNAs showed expression in CD49f-positive cells at least twice as high as in CD49f-negative cells. The expression of the sequenced miRNAs was confirmed by qRT-PCR analysis, and nine differentially expressed miRNAs were selected, including four miRNAs (miR-1468, miR-126-5p, miR-126-3p and miR-10b) with higher read numbers in CD49f-positive cells and 5 miRNAs (miR-9, PC-3p-16208, miR-34c, PC-5p-627 and miR-449b) with higher read numbers in CD49f-negative cells. The qRT-PCR analysis indicated that the high-throughput sequencing data were accurate and reliable.

Several studies have found that some miRNAs participate in spermatogonia self-renewal and spermatogenesis, including miR-221, miR-21, the miR-449 cluster and miR-34b/c. miR-221 plays a crucial role in maintaining the undifferentiated state of mammalian spermatogonia through repression of KIT expression [Yang et al., 2013]; miR-21 is important in maintaining the spermatogonia population and for spermatogonia self-renewal [Niu et al., 2011]. miR-34c regulates the differentiation of mouse embryonic stem cells into male germ-like cells through RARg and promotes SSC differentiation by targeting Nanos2 [Zhang et al., 2012; Yu et al., 2014]. The expression pattern of the miR-449 cluster resembles that of miR-34b/c during spermatogenesis, the miR-449 cluster might have the same roles in the regulation of male germ cell development as miR-34b/c [Bao et al., 2012]. We found that the miRNAs specific for spermatogonial stem and premeiotic cells, such as miR-221, miR-23a, miR-29b, miR-24, miR-29a, miR-199b, miR-199a, miR-27a, miR-21 and let-7a, let-7e, miR-29c [Smorag et al., 2012], were expressed more highly in CD49f-positive testicular cells. These results suggested that the purified CD49f-positive testicular cells might be a population of dairy goat spermatogonia.

The bioinformatics analysis of target genes of differently expressed miRNAs was useful to further understand the physiological functions and biology processes involving these miRNAs during spermatogonia self-renewal and spermatogenesis. For the miRNAs upregulated in CD49f-positive cells, the GO enrichment analysis from biological processes showed that 40 miRNA target genes were involved in transcription, cell-cycle and DNA damage processes, and the KEGG Pathway annotation showed that 10 target genes were involved in the cell-cycle pathway. The cell-cycle analysis of CD49f-positive and CD49f-negative testicular cells had shown that most CD49f-positive testicular cells were in G0/G1 phase and that more than 80% CD49f-negative testicular cells were in G0/G1 or G2/M phase. This suggested that differentially expressed miRNAs and their target genes were involved in cell-cycle regulation. The cell-cycle progression of undifferentiated spermatogonia is regulated by multiple factors, including transcription factors that regulate the expression of target genes during animal development, such as Oct-4, a POU transcription factor. In the testis, Oct-4 is down-regulated at the entry of the germ cells into the committed programme of spermatogenetic proliferation rather than at the entry into meiosis phase [Pesce et al., 1998]. It was demonstrated that spermatogonia were maintained undifferentiated and with stem-cell capacity by suppressing DNA damage-induced cell-cycle arrest [Takubo et al., 2008] and that the primary defect of spermatogonia differentiation was at the meiotic G2/M transition [Eberhart et al., 1996]. DNA damage is a continuous, random process, and in both sexes, including some undamaged germ cells, and cells with non-lethal levels of damage, it survives the mitotic proliferation and enters meiosis before succumbing to damage acquired during the meiotic stage [Hsia et al., 2003]. The pre-meiotic loss of germ cells with damaged DNA is greater in the male because rapidly cycling cells are particularly susceptible to the deleterious effects of DNA damage on DNA replication [Takubo et al., 2008]. miRNA expression patterns can be developmentally regulated, tissue specific, or steadily expressed in the whole organism, and they are considered to play important roles in cell proliferation, apoptosis, and differentiation [Miska, 2005]. Our comparative miRNAome data of CD49f-positive and CD49f-negative testicular cells provides insights into the mechanism of SSCs' self-renewal and spermatogenesis.

In summary, for the first time, we analysed the miRNA expression profiles in goat CD49f-positive and CD49f-negative testicular cells by high throughput Illumina sequencing. Stem-loop qRT-PCR analysis confirmed the expression of these miRNAs in the testis. The CD49f surface marker was primarily localised in the plasma membrane of goat spermatogonia-like cells, and the miRNAs and marker genes specific to spermatogonial stem cells (SSCs) in testis had a higher level of expression in CD49f-positive testicular cells, including miR-221, miR-23a, miR-29b, miR-24, miR-29a, miR-199b, miR-199a, miR-27a, and miR-21 and CD90, Gfra1 and Plzf. The GO term and KEGG pathway analyses for the predicted miRNA targets further illustrated the likely roles of these miRNAs during spermatogenesis. This study suggests that CD49f could be used to enrich the dairy goat spermatogonia-like cells and provides a useful resource for the further elucidation of the regulatory role of miRNAs in SSCs' self-renewal and spermatogenesis.

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