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Original Article

Expression profile analysis of microRNAs during the oestrous cycle of Qira black sheep

Xi Chen[1,a] Hanying Chen[2,a] Song Jiang[1] Hong Shen[1] Chaocheng Li[1] Xiancun Zeng[1,3*]

Abstract

Ovarian follicular development is closely regulated by a multitude of genes and specific non-coding RNAs (ncRNAs), which are typically altered during the oestrous cycle. Identification of the microRNAs (miRNA) expression profiles in sheep ovaries during the oestrous cycle is a prerequisite to further understanding their functional status. This study presents a genome-wide analysis of miRNAs from three groups of Qira black sheep: oestrus (day 0), dioestrus (day 10) and pro-oestrus (day 14). A total of 545 conserved miRNAs and 206 novel miRNAs were identified. Furthermore, 229 differentially expressed miRNAs were screened in three comparisons and 16 miRNAs were found to be differentially expressed in all three different ovarian phases. 63 and 60 miRNAs were downregulated and 46 and 69 miRNAs were upregulated in dioestrus and pro-oestrus ovarian phases compared with the oestrus phase, respectively. Luciferase reporter assays indicated that ovine STAT3 was a target of miR-125b with two binding sites in the 3′UTR. In summary, the miRNA profiles show the dynamic nature of ovarian miRNAs during the oestrous cycle and will help to further understand the role of miRNAs in the regulation of the oestrous cycle, follicular development and ovarian function in sheep.

Keywords: ovine, ovary, differentially expressed miRNAs, development, STAT3

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Introduction

During the processes of the oestrous cycle are follicle cycle through development, maturation, ovulation, luteinization, and luteal tissue regression (McBride *et al.,* 2012; Ginther *et al.,* 2015). Follicular development and atresia are influenced by numerous extra- and intrafollicular regulators and transcriptional genes have been reported to play important roles in various ovarian functions (Scaramuzzi *et al.,* 2011). The screening of transcriptional genes that are involved in the regulation of the sheep oestrous cycle will have theoretical and practical significance for anoestrus, abnormal oestrus and the improvement of sheep fecundity. However, knowledge about the non-coding RNA (ncRNA) regulation in the oestrous cycle and follicle development is limited.

MicroRNAs (miRNA) are small endogenous, highly conserved ncRNAs, with a length of approximately 22 nucleotides that show spatiotemporal and tissue-specific expression patterns in animals (Lagos-Quintana *et al.,* 2001). They can interact with the 3′UTRs of their target mRNAs and thus regulate mRNA expression by promoting the degradation or repressing the translation of the target mRNA or by enhancing translation at the post-translation stage (Carrington and Ambros, 2003; Vasudevan *et al.,* 2007). The results of extensive studies of sheep miRNAs show that miRNA plays an important role in oogenesis (Yang *et al.,* 2019), follicular/luteal development (Donadeu *et al.,* 2012; McBride *et al.,* 2012), granulosa cell function (Yao *et al.,* 2018), embryo development (Wu *et al.,* 2016), regulation of seasonal oestrus (Di *et al.,* 2014; Yang *et al.,* 2018), prolificacy traits (Shen *et al.,* 2015; Miao *et al.,* 2016; Miao *et al.,* 2017; Pokharel *et al.,* 2018) and other biological processes. These studies indicate that miRNAs might play an important role in ovary follicular development, ovulation and fertility in sheep.

In Northwest China, the Qira black sheep is a renowned local breed due to its high prolificacy, year-round oestrous and high-quality lamb skin (Shen *et al.,* 2015). In this study, the expression profiles of miRNAs in Qira black sheep ovaries among three separate phases (dioestrous, pro-oestrus, and oestrous) of the oestrous cycle were compared using Solexa sequencing technology. A number of important miRNAs involved in the regulation of the oestrous cycle were further studied. The results will be helpful to better understanding the regulation of the oestrous cycle, follicle development and ovulation by miRNAs in sheep.

Materials and Methods

Animal and ovary collection during the oestrous cycle: Nine healthy Qira black sheep (3–4 years of age and with 35-40 kg body weight) were purchased from the Qira Sheep Breeding Farm, in Qira County of Xinjiang Province, China. All sheep were reared under the same conditions. All experimental procedures were approved (A2016-085) by the Ethics Committee for Animal Experimental of the First Affiliated Hospital, Shihezi University School of Medicine. All methods were implemented in accordance with relevant guidelines and regulations.

Oestrus was assessed via teaser ram and vaginal examination and the oestrous cycle of experimental ewes was confirmed via the records of two consecutive oestrous cycles. To achieve homogenization, vaginal sponges (Sansheng Pharmaceutical, Ningbo, China) infused with synthetic progesterone were used to synchronize the oestrous cycles of all nine Qira black sheep. Sponges remained in place for a total of 11 days. Two days after withdrawal of the vaginal sponge, all sheep entered oestrus, which was confirmed with a teaser ram and via vaginal examination. After a second natural oestrus had been diagnosed, three ewes were slaughtered at oestrus (day 0, named Qira-E), dioestrus (day 10, named Qira-D) and pro-oestrus (day 14, named Qira-P) to collect ovarian follicles and follicular peripheral tissue , respectively.

Small RNA library construction and Solexa sequencing: Total RNA from the ovarian tissue of each sheep was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. The quality of the total RNA was examined by Agilent 2100 Bioanalyzer and an RNA integrity number (RIN) range of 7.7-8 was considered to indicate qualified RNA. After isolation and purification of small RNA molecules shorter than 35 nt in length, the 5′ and 3′ solexa adaptors（Illumina, NEB, USA） were ligated to the small RNA pool. Homology comparison was performed using NCBI's blastn software and then the genome sequences with higher homology were analyzed for secondary structure and, finally, miRNAs that could form a stem loop structure were identified as new miRNAs. Appropriate PCR cycles were used to construct a sequencing library, which was sequenced on an Illumina Genome Analyzer by Novogene (Beijing, China).

Sequencing data analysis: The adaptors, low quality reads and contaminated sequences were removed to obtain clean reads; then, the number and length of the clean reads were measured. The clean reads were blasted against both the GenBank database and the NCBI Rfam database to annotate small RNA sequences and the *Ovis aries* genome (Oar_v3.1) to analyse their expression and distribution within the genome using Short oligonucleotide alignment program (SOAP) v1.11 software (Li *et al.,* 2008). Clean reads were searched against miRBase 22.0 to identify conserved miRNAs, and then, the remaining unmapped reads were used to identify putative miRNAs.

Mireap software was used to predict the target genes of the miRNAs (Bentwich, 2005; Yuan *et al.,* 2013). Finally, GO annotation and KEGG pathway analysis were performed to investigate the biological functions and pathways related to putative target genes.

MiRNA differential expression analysis: The differentially expressed miRNAs during the three stages (dioestrus, pro-oestrus and oestrus) were identified using the DESeq 2 program (Anders and Huber, 2010). After normalising the miRNAs expression level in each library, the threshold values of

fold-change and p-value adjusted (padj) were calculated to identify differentially expressed miRNAs. These were identified if their threshold values met the following criterion: padj < 0.05.

Validation of miRNA target genes: Based on target gene prediction using the RNA22 program, the Dual-luciferase reporter assays was used to determine whether miR-125b could directly control the signal transducer and ovine regulate activator of transcription 3 (STAT3).To clone the 3′UTR of STAT3 (from 2544 to 3719, XM_004012925.1), which contains the putative miR-125b binding sites (from 2643 to 2665 and from 3460 to 3489, XM_004012925.1) into a dual-luciferase reporter vector pmiR-RB-Report (Ribobio, China) to generate pmiR-RB-Report + STAT3 3′UTR (named STAT3-WT), the 3′UTR was PCR amplified using the following primers: 3′UTR-F 5-GGCGG**CTCGAG**TCACTTTAGCTATCTGTCAGC-3 and 3′UTR-R 5-AAT**GCGGCCGC**ATGCTACCTGTG TTCTGAGAT-3 (product size 1175 bp), where XhoI and NotI sites are underlined and in bold, respectively. Based on recombinant plasmids of pmiR-RB-Report-STAT3 3′UTR, mutations were introduced into both putative miR-125b binding sites located in the 3′UTR of STAT3 using the overlap PCR method. The following primer sequences were used to construct the pmiR-RB-Report + binding sites in STAT3 3′UTR mutants: binding site 1 (from 2659 to 2664, TCAGGG mutated to AGTCCC, and named STAT3-Mut1), STAT3-Mut1-F: 5-GACTGTGAAGTCCCGATGTGGA AGGGGCGGTG-3, STAT3-Mut1-R: 5-TCCACATCGG GACTTCACAGTCAAGGAGTGCG-3, binding site 2 (from 3483 to 3489, TCAGGGA mutated to AGTCCCT, and named STAT3-Mut2), STAT3-Mut2-F: 5-AGTTGAGCAGTCCCTATATGGTTCTTATCCCAG-3 and STAT3-Mut2-R: 5-AACCATATAGGGACTGCT CAACTAGACCCTTGC-3. HEK293T cells were seeded into the wells of a 96-well plate for 24 h. The 293T cells were injected with STAT3-WT + NC24 (micrON mimic NC #24, ribobio, China; miRNA mimics control), STAT3-WT + oar-miR-125b mimics(ribobio, Guangzhou,China), STAT3-Mut1 + NC24, STAT3-Mut1 + oar-miR-125b mimics, STAT3-Mut2 + NC24, and STAT3-Mut2 + oar-miR-125b mimics using Lipofectamine 2000 (Invitrogen, USA). After 48 h of transfection, the luciferase activity was independently measured three times using the dual-luciferase reporter assay system (Promega, USA).

Results

Small-RNA sequencing of Qira black sheep ovaries during the oestrous cycle: To identify differentially expressed miRNAs involved in the sheep oestrous cycle, nine small-RNA libraries (three libraries per group) that had been prepared from individual ovary samples collected from Qira black sheep at oestrus, dioestrus and pro-oestrus during the oestrous cycle were sequenced. On average, more than 13 million raw sequences and 13 million clean reads were obtained from each sample (Table 1). The most common read lengths ranged between 21 and 23 nucleotides, corresponding to the length of mature miRNAs. Clean reads with a length of 18-35 nucleotides were aligned against the latest sheep genome sequence using SOAP software (Li *et al.*, 2008). Then, genome-matched small RNA tags were clustered into several sRNA categories (Table 2). More than 11.8 million reads perfectly matched the sheep genome (Table 2). Conserved miRNAs, predicted novel miRNAs and unannotated sequences accounted for 60.60% (7,160,997 reads), 0.016% (1,927 reads) and 35.79% (4,228,671 reads) of the genome-matched reads in the ovarian libraries, respectively (Table 2).

A total of 67,302 unique sequences (104,628,258 reads) and 3,421 unique sequences (29,644 reads) were annotated as known miRNA and novel miRNA candidates, respectively (Table S1). 545 conserved miRNAs and 206 novel miRNAs were identified in this study (Tables S1 and S2). Strong positive correlations (R^2 > 0.975) were found between the nine samples based on miRNA expression levels (Fig. 1). A number of the 10 most abundant miRNAs in the oestrous ovary of Qira black sheep (miR-99a, miR-26a, miR-143, miR-21, let-7f, and let-7g; see Table 3) were also identified as highly abundant in the oestrous ovaries of Hetian sheep and Small tail han sheep using Illumina technology (Di *et al.*, 2014; Shen, *et al.*, 2015). Eight miRNAs including miRNA-99a, miRNA-148a, miRNA-26a, miRNA-10b, and miRNA-21 were highly expressed in all three periods; miRNA-27b was highly expressed only in the Qira-D period and let-7i was highly expressed only in the Qira-P period; and miRNA-125b was highly expressed in the two periods Qira-D and Qira-E.

Table 1 Summary of small RNA sequencing data obtained from sheep ovarian tissue using Solexa sequencing.

Sample	total reads	N% > 10%	low quality	5 adapter contamine	3 adapter null or insert null	with ployA/T/G/C	clean reads
Qira-D1	11,609,388	312	44,282	274	140,972	2,711	11,420,837
Qira-D2	12,813,688	421	49,790	607	134,844	4,596	12,623,430
Qira-D3	12,088,568	358	47,816	345	64,676	3,165	11,972,208
Qira-P1	12,929,907	199	33,842	222	83,893	4,096	12,807,655
Qira-P2	13,929,907	542	23,575	132	63,262	3,273	13,839,123
Qira-P3	13,200,653	606	23,440	290	89,798	5,658	13,080,861
Qira-E1	13,708,248	265	38,717	144	87,676	3,537	13,577,909
Qira-E2	13,430,338	260	42,412	186	77,913	3,644	13,305,923
Qira-E3	14,641,423	1,142	44,858	258	95,466	5,399	14,494,300
mean	13,150,236	456	38,748	273	93,167	4,009	13,013,583

Table 2 Flowing results of data filtration and distribution of sequenced sRNA from sheep ovaries.

Types	Qira-D1	Qira-D2	Qira-D3	Qira-P1	Qira-P2	Qira-P3	Qira-E1	Qira-E2	Qira-E3	mean
Total sRNA	11,320,513	12,427,433	11,846,522	12,429,655	13,588,868	12,646,195	13,398,177	13,101,435	14,300,612	12,784,379
Mapped genome	10,475,231	11,388,392	10,930,200	11,424,717	12,646,220	11,593,848	12,445,044	12,206,478	13,233,217	11,815,927
Known_miRNA	6,472,503	6,565,309	6,906,632	7,143,328	7,781,151	6,709,915	7,347,090	7,642,530	7,880,518	7,160,997
RRNA	4,476	10,700	6,509	9,604	12,826	20,701	8,543	9,638	12,341	10,593
TRNA	164	350	213	516	171	609	195	133	280	292
SnRNA	2,259	3,571	2,476	2,849	2,495	3,559	3,023	3,827	3,696	3,084
SnoRNA	162,617	225,021	127,572	74,220	53,610	92,168	82,330	93,367	172,387	120,366
Sepeat	31,287	64,478	37,796	53,552	38,789	68,689	44,970	68,159	80,120	54,204
Novel_miRNA	990	1,754	1,628	3,017	2,162	2,038	1,842	1,889	2,019	1,927
Exon:+	48,240	77,440	56,939	69,207	48,904	66,100	48,342	57,207	77,496	61,097
Exon:-	6,291	13,606	8,273	14,018	11,725	21,781	12,111	13,909	17,514	13,248
Intron:+	41,662	80,882	52,680	82,278	74,697	116,757	70,484	80,343	94,394	77,131
Intron:-	41,201	93,445	62,463	87,930	83,944	132,599	67,273	83,866	106,140	84,318
Other	3,663,541	4,251,836	3,667,019	3,884,198	4,535,746	4,358,932	4,758,841	4,151,610	4,786,312	4,228,671

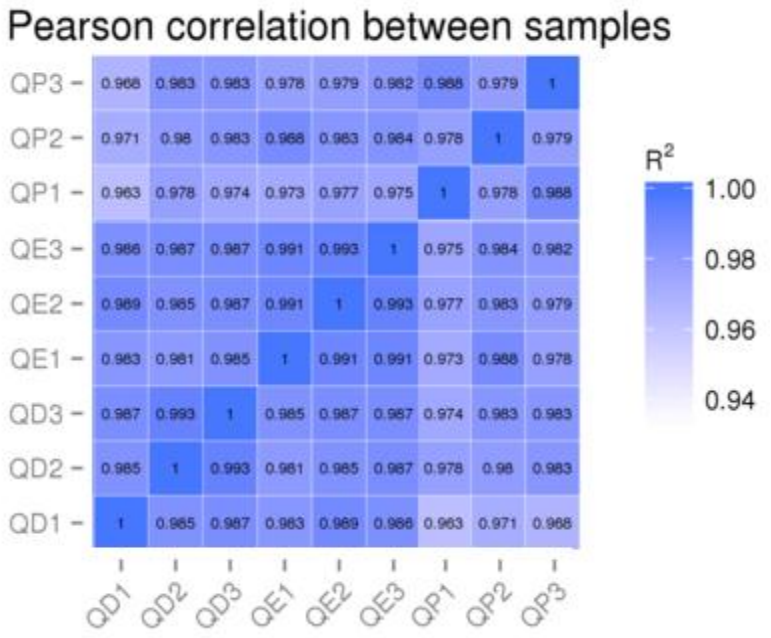

Figure 1 The correlations between the nine samples based on miRNAs level of expressions. R^2: square of the Pearson correlation coefficient.

Table 3 Highly expressed miRNAs (i.e., the top 10) in the ovarian libraries of the Qira black sheep oestrous cycle.

Qira-D		Qira-P		Qira-E	
miRNA	**Count**	**miRNA**	**Count**	**miRNA**	**Count**
miR-99a	924,182	miR-148a	1,622,286	miR-148a	1,752,226
miR-148a	918,768	miR-99a	976,664	miR-99a	1,252,245
miR-26a	786,105	miR-143	598,023	miR-26a	797,140
miR-26c	786,059	miR-26a	570,250	miR-26c	797,107
miR-143	590,561	miR-26c	570,223	miR-143	566,776
miR-10b	439,991	miR-21	492,338	miR-10b	440,426
miR-125b	267,960	miR-10b	281,038	miR-21	214,042
miR-21	223,522	let-7i	220,020	let-7f	198,067
miR-27b	166,059	let-7g	204,902	let-7g	193,547
let-7g	165,665	let-7f	202,284	miR-125b	172,541

***Differential expression analysis of miRNAs*:** The results showed that 109, 129, and 152 differentially expressed miRNAs were screened between oestrus vs dioestrus, oestrus vs pro-oestrus and pro-oestrus vs dioestrus, respectively (Table S3, Fig. 2). 63 miRNAs were upregulated and 46 miRNAs were downregulated in dioestrus compared to oestrus. 60 miRNAs were upregulated and 69 miRNAs were downregulated in pro-oestrus compared to oestrus. 83 miRNAs were upregulated and 69 miRNAs were downregulated in pro-oestrus compared to dioestrus. There were eight miRNAs that were highly expressed during all three periods: diestrus, proestrus and estrus. Among them, the expression trends of miR-99a and miR-148 were always increasing; the expression trends of miR-26a, miR-26c, and miR-10b were first decreased

and then increased; and the expression trends of miR-143, miR-21, and let-7g were first increased and then decreased. (Table 3)

Of the differentially expressed miRNAs, 16 were co-expressed during all three comparisons, while 28, 29 and 27 miRNAs were only differentially expressed in the oestrus vs dioestrus, oestrus vs pro-oestrus, and pro-oestrus vs dioestrus, respectively (Table 4, Fig. 2). Furthermore, 36, 61, and 80 differentially expressed miRNAs were co-expressed in the oestrus vs dioestrus and oestrus vs pro-oestrus, oestrus vs dioestrus and pro-oestrus vs dioestrus, oestrus vs pro-oestrus and pro-oestrus vs dioestrus, respectively (Table S4, Fig. 2).

Table 4 Co-expressed miRNAs among oestrus vs dioestrus, oestrus vs pro-oestrus and pro-oestrus vs dioestrus.

sRNA	Qira_E_readcount	Qira_P_readcount	Qira_D_readcount	Qira-E/ D	Qira-E/ P	Qira-P/ D
let-7i	144,810.90	203,422.14	126,909.07	0.31	-0.33	0.66
miR-101	55,565.20	36,528.21	99,319.63	-0.68	0.71	-1.37
miR-106b	1,631.81	1,387.41	2,810.99	-0.63	0.37	-0.98
miR-148a	1,778,894.00	1,512,370.01	984,222.10	0.90	0.37	0.58
miR-19b	1,391.87	954.51	4,162.65	-1.29	0.62	-2.01
miR-2478	172.68	313.86	92.12	0.90	-0.68	1.57
miR-320a	7,300.09	4,219.54	8,816.61	-0.14	0.86	-0.99
miR-342	4,623.73	3,477.99	6,230.10	-0.29	0.54	-0.82
miR-362-3p	41.91	25.63	74.97	-0.65	0.77	-1.44
miR-450b	3,933.11	5,941.96	3,167.64	0.43	-0.43	0.87
miR-486	156.79	290.04	94.06	0.77	-0.67	1.48
miR-493	964.84	1,632.65	762.53	0.45	-0.57	1.02
miR-497	1,741.29	2,512.83	3,674.68	-0.90	-0.38	-0.53
miR-532	38,972.29	22,030.89	29,033.94	0.54	0.95	-0.39
miR-708	1,202.56	984.03	1,625.64	-0.30	0.42	-0.70
novel_158	33.46	96.08	14.77	0.97	-1.18	2.27

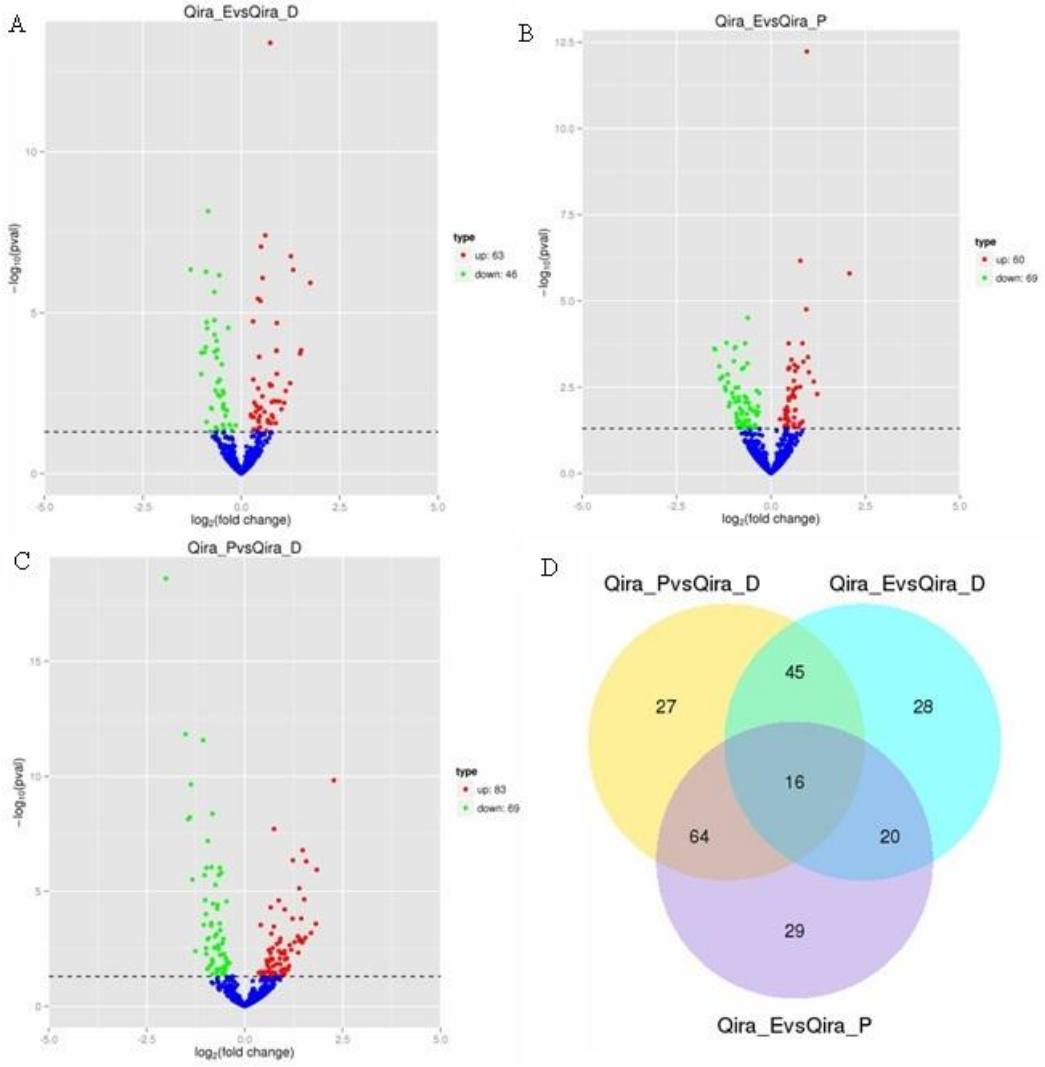

Figure 2 Differential expression analysis of miRNAs among the oestrus, dioestrus and pro-oestrus of the sheep oestrous cycle. (A) Oestrus vs dioestrus. (B) Oestrus vs pro-oestrus. (C) Pro-oestrus vs dioestrus. (D) Venn diagram for the differentially expressed miRNAs in the three comparisons.

Similarly, several previous studies have identified the differentially expressed miRNAs that are involved in mouse follicular development (Yang *et al.*, 2013), bovine follicle development (Sontakke *et al.*, 2014; Zielak-Steciwko *et al.*, 2014) and sheep and goat follicular-luteal transition (Zhu *et al.*, 2016; Wang *et al.*, 2018). It has been shown that miRNAs play critical roles in almost all ovarian biological processes, such as follicular development and atresia (Donadeu *et al.*, 2012; Navakanitworakul *et al.*, 2016; Worku *et al.*, 2017; Zhang *et al.*, 2017[a]), granulosa cell proliferation (Yan *et al.*, 2012; Sirotkin *et al.*, 2014; Andreas *et al.*, 2016; Gebremedhn *et al.*, 2016), apoptosis (Yang *et al.*, 2012; Sirotkin *et al.*, 2014; Gebremedhn *et al.*, 2016) and steroidogenesis (Dai *et al.*, 2013).

KEGG pathway and GO annotation analyses could yield a better understanding of the signalling pathway and biological functions (cellular components, biological processes and molecular functions) of target genes of the differentially expressed miRNAs. However, the results showed no significantly enriched pathways and GO terms. The reason for the absence of significantly enriched pathways and GO clauses, we speculate, is that the miRNAs in this experiment may not have the bias of kegg pathway and GO terms; it may also be because the differential genes screened out by the fixed threshold we used were too few and were scattered in various types of pathways, were unable to form enrichment and naturally had no significantly enriched pathways and GO terms.

Identification of STAT3 as a target gene of miR-125b: In previous studies, miR-125 has been studied mostly on human diseases (Yao *et al.*, 2013; Peng *et al.*, 2013) and also on goat hair follicles (Yuan *et al.*, 2013) but there are a few studies on miR-125 in sheep ovaries. The present study showed that miR-125b was significantly differentially expressed in oestrus vs dioestrus and pro-oestrus vs dioestrus. In addition, miR-125b was expressed at higher levels in the ovaries of Qira black sheep than in those of Hetian sheep (Shen *et al.*, 2015) and in follicular tissues compared to luteal tissues (McBride *et al.*, 2012). However, it has been reported that STAT3 might be involved in cattle granulosa cell death, follicular atresia (Gasperin *et al.*, 2015) and mouse granulosa cell apoptosis (Wang *et al.*, 2016). To experimentally validate sheep STAT3 as a miR-125b specific target, luciferase reporter plasmids of the wild type STAT3 3′UTR were constructed as well as mutants of both putative miR-125b binding sites. After over-expression of miR-125b in HEK293T cells, luciferase activity indeed decreased significantly ($p < 0.01$) compared to cells carrying the STAT3-WT + NC24. Furthermore, luciferase activity decreased significantly ($p < 0.05$) compared to cells carrying the STAT3-Mut1 + NC24 and STAT3-Mut2 + NC24 (Fig 3). All of this data indicates that miR-125b targets STAT3 by binding to the 3′UTR (two binding sites) (Fig 3).

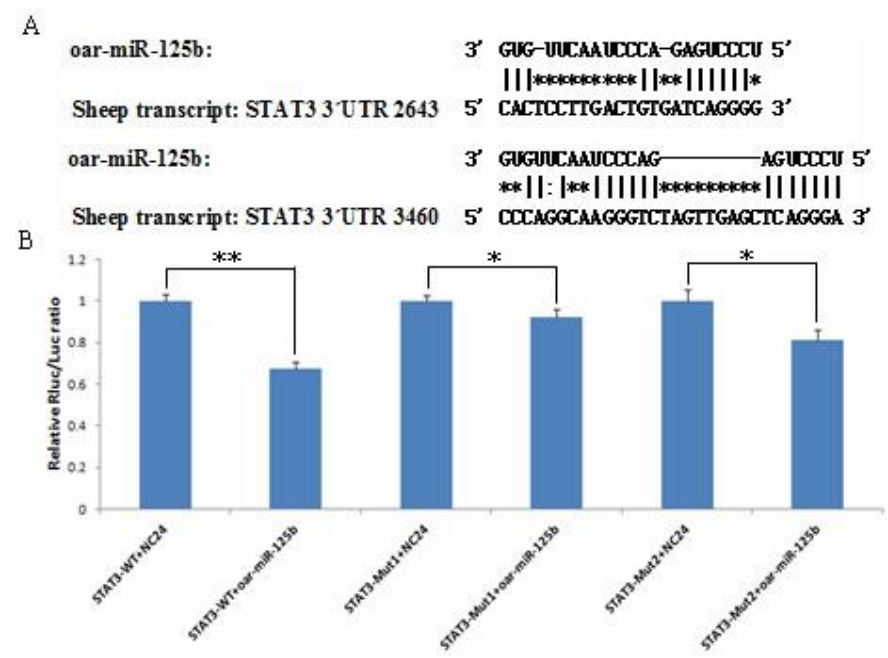

Figure 3 Identification of STAT3 as a target gene of miR-125b in HEK293T cells. (A) Putative binding sites for sheep (oar) miR-125b and the 3′UTR of the STAT3 gene. (B) HEK293T cells were infected STAT3-WT + NC24, STAT3-WT + oar-miR-125b mimics, STAT3-Mut1 + NC24, STAT3-Mut1 + oar-miR-125b mimics, STAT3-Mut2 + NC24, and STAT3-Mut2 + oar-miR-125b mimics. After 48 h, luciferase assays were performed. *$p < 0.05$, **$p < 0.01$, compared to the NC24 group.

Discussion

According to the results obtained in the present study, these miRNAs were also highly expressed in Qira black sheep ovaries at different stages during the oestrous cycle. This is consistent with the results in ovine ovaries at different stages during the breeding season (Di *et al.*, 2014). This conserved high level of expression across the oestrous cycle suggests that these miRNAs may play important roles in sheep ovarian function. In previous studies, miR-99a was expressed at significantly higher levels in follicular tissues of sheep compared to their luteal tissues (McBride *et al.*,

2012). miR-99a was identified as being able to regulate the proliferation and apoptosis of human granulosa cells in the polycystic ovary syndrome via targeting IGF-1R (Geng *et al.,* 2019). miR-143 in particular plays a critical role in the FSH-induced oestradiol production, mouse granulosa cell proliferation (Zhang *et al.,* 2017[b]) and porcine granulosa cell apoptosis (Du *et al.,* 2016). Furthermore, miR-26a might play a role in sheep follicular development (Zhang *et al.,* 2017[a]) and miR-26a-5p has been reported to be critical for the proliferation of chicken theca cells (Kang *et al.,* 2017).

Co-expressed miRNAs help to identify candidate miRNAs associated with ovine reproductive characteristics, such as follicular development and granulosa cell function. For example, miR-378 and miRNA-383 are critical for the regulation of steroid synthesis and oocyte maturation (Xu *et al.,* 2011; Yin *et al.,* 2012; Pan *et al.,* 2015). miR-26b, miR-10b, miR-224, and miR-21 regulate the proliferation or apoptosis of granulosa cells (Carletti *et al.,* 2010; Yao *et al.,* 2010[a]; Lin *et al.,* 2012; Peng *et al.,* 2016; Liu *et al.,* 2016). let-7 and miR-106 are necessary for the normal development of the bovine oocytes (Miles *et al.,* 2012). miR-202 was upregulated in bovine large healthy follicles compared to small follicles and large atretic follicles have been suggested to play roles in follicular selection and dominance (Sontakke *et al.,* 2014). It has also been shown that miR-378 may inhibit apoptosis of luteal cells by inhibiting IFNGR1(Ma *et al.,* 2011).

The present study also identified STAT3, a target gene of miR-125b. Similarly, a previous study showed that miRNA-125b-5p suppresses the development of human hypothyroidism by targeting STAT3 (Liu *et al.,* 2018). Moreover, miR-125b regulates yak granulosa cell apoptosis by targeting BMPR1B (Yao *et al.,* 2018), and miR-125b was upregulated in rat granulosa cells that were exposed to FSH for 12 h (Yao *et al.,* 2010[b]). In summary, it is plausible that miR-125b plays a substantial role in sheep follicle development, granulosa cell proliferation and apoptosis, and other ovarian functions. However, the functional status of miR-125b requires further study.

In conclusion, this study provides the first small RNA sequencing analysis of ovarian tissues during the Qira black sheep oestrous cycle. Furthermore, 229 differentially expressed miRNAs were identified in the three comparisons (oestrus vs dioestrus, oestrus vs pro-oestrus and pro-oestrus vs dioestrus) and 16 differentially expressed miRNAs were co-expressed in these three comparisons. These results reflect changes in miRNA expression in ovarian tissues that are likely involved in the regulation of the sheep oestrous cycle and follicular development. In addition, this study identified the interplay between miR-125b and STAT3. The results of this study will expand the database on sheep miRNAs and are useful for future functional studies of miRNAs involved in follicular development, ovulation and the function of granulosa cells. The limitation of this experiment is that there is no study that rises to the cellular level. We should further verify the function of granulosa cells in future studies, etc., and we can also select more target genes of some differentially expressed miRNAs for validation and expand their validation range.

Conflict of interest: None of the authors has any conflict of interest to declare.

Data availability statement: Data sharing is available with this article.

Author contributions: Xiancun Zeng designed the study, conducted the experiments, analysed the data and drafted the manuscript. Hanying Chen and Xi Chen designed the study and drafted the manuscript. Song Jiang, Hong Shen and Chaocheng Li conducted parts of the experiments and collected samples.

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