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Characterization and expression analysis of microRNAs in Qira black sheep and Hetian sheep ovaries using Solexa sequencing

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ABSTRACT. The role of microRNAs (miRNAs) in the regulation of mammalian reproduction has been demonstrated previously. However, only a few studies have assessed the role of miRNAs in the reproduction processes of sheep. The elucidation of miRNA expression profiles in the ovaries of different sheep breeds representing fecundity extremes will be useful in understanding the roles of miRNAs in sheep reproduction. In this study, two small RNA libraries were constructed from ovary tissue taken from Qira black sheep and Hetian sheep during the estrous period and then sequenced using the Solexa sequencing method. We obtained 9,565,212 and 9,563,426 high-quality reads from Qira black sheep and Hetian sheep, respectively. In total, 531 miRNAs, including 98 putative miRNAs, were identified. Among the conserved miRNAs, 125 known miRNAs were significantly differentially expressed in the Qira black sheep and Hetian sheep libraries, with 24 upregulated and 101 downregulated in the Hetian sheep compared to the Qira black sheep. Four differentially expressed miRNAs were analyzed using real-time

quantitative PCR to validate the reliability of the Solexa sequencing results. These results provide a foundation for future research on the regulation of miRNAs in sheep fertility and enrich the sheep miRNA databases.

Key words: Sheep; MicroRNA; Solexa sequencing; Ovary; Expression profiles

INTRODUCTION

MicroRNAs (miRNAs) are a group of endogenously non-coding small RNA molecules, approximately 22 nucleotides (nt) in length, which negatively regulate gene expression through complementary base pairing with target mRNAs (Kim, 2005). A broad range of biological processes, such as cell differentiation, proliferation and apoptosis, tissue morphogenesis, fertility, metabolism, and immune response, have been shown to be regulated by miRNAs (Ambros, 2004; Plasterk, 2006; Shivdasani, 2006; Sun et al., 2012; Hossain et al., 2012a). As a major regulator of gene expression, several studies have used Solexa sequencing to demonstrate that miRNAs play specific roles in ovarian function and ovulation (Baley and Li, 2012; Hossain et al., 2012b). For example, several studies have been undertaken to identify the distinct miRNAs in the ovaries of pregnant and non-pregnant goats (Zhang et al., 2013), 2-week-old and adult mouse ovaries (Ro et al., 2007), adult bovine ovaries (Hossain et al., 2009), bovine fetal ovaries (Tripurani et al., 2010), and new-born mouse ovaries (Ahn et al., 2010). Zhang et al. (2013) identified 624 miRNAs using deep sequencing of goat ovary tissue representing both the pregnant and non-pregnant stages, and 407 conserved miRNAs were differentially expressed. Additionally, a large body of evidence is available on the involvement of miRNAs in follicular development (Kim et al., 2010; Donadeu et al., 2012; McBride et al., 2012), the function of granulosa cells (Sirotkin et al., 2010; Xu et al., 2011), and embryo development (Suh et al., 2010; Baley and Li, 2012; Hossain et al., 2012a). A similar study using a microarray approach showed 11 differentially expressed miRNAs in ovarian tissue of single-bearing and biparous ewes of the Bamei mutton sheep, suggesting that these miRNAs may be related to sheep follicular development and litter size (Qi, 2013). These findings suggest that identifying miRNA expression profiles in ovary tissues is a valuable approach for identifying the miRNAs involved in follicular development, ovulation, and, thus, sheep fertility.

Qira black sheep and Hetian sheep are two local breeds in the Hetian region of northwest China. The former is characterized by high fecundity with an average lambing rate of 215.5% (Bai, 2007), while the latter is a low prolificacy breed with an average lambing rate of 102.5% (Zeng et al., 2010). The difference in fertility of these breeds may be an effect of ovarian function or ovulation rate (Bai, 2007). Significant genetic differences between these two breeds have provided increasing interest in the identification and utilization of major prolificacy miRNAs in these sheep. However, much less is known about the roles of miRNAs in sheep reproduction processes (Donadeu et al., 2012; McBride et al., 2012; Qi, 2013).

In the present study, we performed Solexa sequencing to analyze ovarian miRNA expression profiles, and to compare differences in miRNA expression, between Qira black sheep and Hetian sheep. Elucidation of the expression patterns of different miRNAs between different breeds will be useful for understanding the roles of miRNAs in reproductive biological processes and will provide basic data for future studies.

MATERIAL AND METHODS

Ethics statement

Qira black sheep and Hetian sheep were purchased from the Qira Sheep Breeding Farm located in Qira County, Hetian region, Xinjiang Province, China. The Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, 2004), and the Regulations for the Experimental Animal Use Management of Shihezi University were used to conduct the experimental research. All ewes were housed in one group under normal conditions of natural lighting and free access to feed. All ewes were killed humanely using anesthesia (xylidinothiazoline, 1 mg/kg; Feilong animal pharmaceutical factory, Heilongjiang, China), and performed by professional workers at the Qira Cattle and Sheep Slaughterhouse.

Ovary collection, library construction, and Solexa sequencing

Five Qira black ewes (high prolificacy individuals) and four Hetian ewes (low prolificacy individuals) of a similar age (3-4 years old) and body size were selected, according to reproduction records, age, and body size. All ewes were checked daily for signs of estrus with a teaser ram. After estrus was diagnosed, all ewes reached spontaneous estrus following an estrous cycle and then ewes were killed between 24 and 36 h after spontaneous estrus was detected. Ovaries were removed immediately after slaughter, and immersed and stored in liquid nitrogen.

Total RNA was isolated from ovarian tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer protocol. The same group of total RNA was pooled with equal quantity of each total RNA type. Briefly, after purification using 15% polyacrylamide gel electrophoresis, molecules less than 35 nt in length had a pair of Solexa adapters ligated to their 5' and 3' termini. Appropriate PCR cycles were performed to produce sequencing libraries, which were the sequenced using the Illumina Genome Analyzer at the Shenzhen Genomics Institute (BGI, Shenzhen, China).

Data analysis

After removing the contaminated sequences, adaptors, and low quality reads, all valid sequences were obtained, and the length and number of unique and total reads were counted. Subsequently, all unique sequences were compared with the Rfam and GenBank database to annotate the sRNA sequences. The clean reads were first aligned against the latest sheep genome (oar 3.1) using the program SOAP (Li et al., 2008), and then searched against the miRNA precursors/mature miRNAs of *Ovis aries* or *Bos taurus* in miRBase 20.0 (http://www.mirbase.org/) to identify conserved miRNAs. Subsequently, the Mireap software (Bentwich, 2005) was used to analyze the remaining unmapped reads to identify the sheep putative miRNAs, and to predict the target genes of the miRNAs with the specific parameter settings described by Yuan et al. (2013). Finally, the main pathways significantly associated with the predicted target gene candidates of the miRNAs were also revealed using KEGG Pathway analysis.

We assessed ovarian miRNA expression levels in Qira black sheep and Hetian sheep to reveal differentially expressed miRNAs. Scatter plot and log2-ratio figures were used to show the expression of miRNAs. The procedures used were as follows: 1) to obtain the ex-

pression of transcripts per million, the expression of each miRNA in Qira black sheep and Hetian sheep was normalized; 2) using formulas described by previous studies (Huang et al., 2011; Sun et al., 2013; Yuan et al., 2013; Zhang et al., 2013), the fold-change and P values were calculated from the normalized expression.

Confirmation of differentially expressed miRNAs using real-time quantitative PCR (qPCR)

Four differentially expressed miRNAs (miR-23b, miR-26a, miR-432, and miR-760-3p) were analyzed using qPCR to verify the Solexa sequencing data. Total RNA was extracted from ovarian tissue of both sheep breeds separately using the mirVana™ miRNA Isolation Kit (Ambion, USA). According to the manufacturer protocol, 1 µg total RNA from each sample was reverse transcribed to cDNA using the NCode™ VILO™ miRNA cDNA Synthesis Kit (Ambion). Real-time qPCR was performed using standard protocols on the Mx3000 System with the NCode™ EXPRESS SYBR® GreenER™ miRNA qRT-PCR Kit (Ambion). In a 20-µL reaction volume, 2 µL cDNA was used for amplification, with 10 µL SuperMix Universal, 0.4 µL specific forward and universal primers, and 8.2 µL water. The reaction was amplified at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s for miR-26a and miR-432, or 61°C for 30 s for miR-23b and miR-760-3p. For each miRNA, all reactions were run in triplicate. The relative amount of each miRNA to U6 snRNA was evaluated using the equation $2^{-\Delta\Delta CT}$ according to the threshold cycle (CT) (Deo et al., 2011).

RESULTS

Overview of the Solexa sequencing data

In order to identify differentially expressed miRNAs in the ovaries of Qira black and Hetian sheep, two small RNA libraries were constructed using Solexa sequencing. First, we obtained 9,565,212 high-quality reads from the Qira black sheep library and 9,563,426 high-quality reads from the Hetian sheep library. After deleting contaminated reads, 9,534,373 and 9,529,095 clean reads remained in the Qira black sheep and Hetian sheep libraries, respectively (Table 1). The size distribution of the reads was similar between the two libraries (Figure 1). Among these sequences, the majority of small RNAs were 21-23 nt in size. Sequences that were 22 nt in length accounted for 57.34 and 57.83% of total sequence reads in the Qira black and Hetian sheep ovarian libraries, respectively, which is the typical size range for Dicer-derived products. In order to analyze the expression and distribution of sequences from the two libraries against the sheep genome, all clean Solexa reads were aligned against the latest sheep genome sequence using SOAP software (Li et al., 2008). A total of 7,554,306 reads and 81,357 unique sRNAs of the Qira black sheep library were perfectly matched to the sheep genome, as well as 7,738,055 reads and 78,137 unique sRNAs of the Hetian sheep library (Table 2). The genome-matched small RNA tags were clustered into several RNA categories (exon_antisense, exon_sense, intron_antisense, intron_sense, miRNA, unannotated, and others; Table 2). Conserved miRNAs accounted for 76.13 and 78.17% of the total sequence reads, and 1.79 and 1.65% of the unique sequence reads, in the Qira black and Hetian sheep libraries, respectively. Furthermore, the highest fraction of unique reads (65.69 and 64.69% in the Qira black and Hetian sheep libraries, respectively) was attributed to unannotated sequences.

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

Type	Qira black sheep library		Hetian sheep library	
	Count	Percent (%)	Count	Percent (%)
Total reads	9,600,000		9,600,000	
High quality reads	9,565,212	100.00	9,563,426	100.00
3' adapter null reads	4,265	0.04	4,709	0.05
Insert null reads	2,087	0.02	1,988	0.02
5' adapter contaminants	16,112	0.17	15,720	0.16
Reads smaller than 18 nt	8,355	0.09	11,900	0.12
PolyA	20	0.00	14	0.00
Clean reads	9,534,373	99.68	9,529,095	99.64

nt = nucleotides.

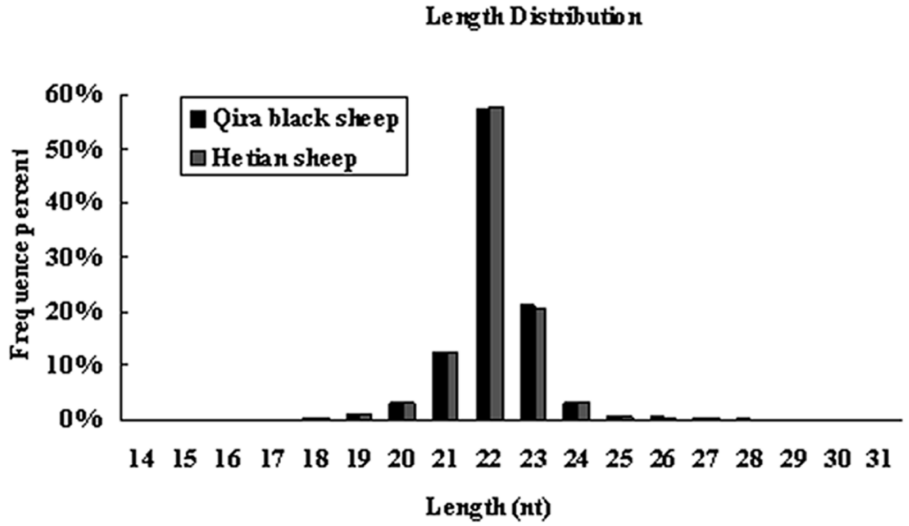

Figure 1. Length distribution of small RNAs in the Qira black sheep (black) and Hetian sheep (gray) libraries.

Table 2. Flowing results of data filtration and the distribution of sequenced small RNAs from sheep ovaries.

Category	Qira black sheep				Hetian sheep			
	Unique reads	Percent (%)	Total reads	Percent (%)	Unique reads	Percent (%)	Total reads	Percent (%)
Clean reads	194,472	100.00	9,534,373	100.00	193,404	100.00	9,529,095	100.00
Match genome	81,357	41.83	7,554,306	79.23	78,137	40.40	7,738,055	81.20
Exon antisense	408	0.21	673	0.01	382	0.20	578	0.01
Exon sense	13,948	7.17	35,064	0.37	16,334	8.45	31,335	0.33
Intron antisense	4,729	2.43	50,240	0.53	4,187	2.16	54,814	0.58
Intron sense	10,903	5.61	23,226	0.24	9,910	5.12	26,667	0.28
miRNA	3,472	1.79	7,258,339	76.13	3,183	1.65	7,448,655	78.17
rRNA	14,446	7.43	95,851	1.01	14,949	7.73	97,650	1.02
repeat	11,245	5.78	22,198	0.23	10,648	5.51	22,074	0.23
snRNA	1,433	0.74	4,010	0.04	1,281	0.66	3,943	0.04
snoRNA	1,679	0.86	7,606	0.08	1,391	0.72	6,514	0.07
tRNA	4,460	2.29	61,934	0.65	6,035	3.12	71,488	0.75
unann	127,749	65.69	1,975,232	20.72	125,104	64.69	1,765,377	18.53

unann = unannotated reads.

Identification of conserved miRNAs

To identify conserved miRNAs in sheep ovaries, the clean reads were aligned against the miRNA precursors/mature miRNAs of *O. aries* or other mammals in miRBase 20.0. A total of 3472 unique sequences (7,258,339 reads) were annotated as miRNA candidates in the Qira black sheep library and 3183 unique sequences (7,448,655 reads) in the Hetian sheep library, while the rest were unannotated (Table 2). Three hundred and ninety seven and 395 conserved miRNAs were identified in the Qira black sheep and Hetian sheep libraries, respectively (**Table S1**). The conserved miRNAs were categorized into two groups based on their hits: the results revealed that 138 miRNAs matched with known sheep miRNAs registered in the miRBase database and 295 miRNAs were conserved among other mammals but have yet to be identified in sheep. As shown in **Table S1**, 359 unique miRNAs (82.91%) were co-expressed in both libraries, and 38 (8.78%) and 36 (8.31%) of these miRNAs appeared to be preferentially expressed in the Qira black sheep and Hetian sheep libraries, respectively, indicating that most of the conserved miRNAs were shared between these two breeds. In the two libraries, the abundance of expression of let-7a, let-7b, let-7c, let-7f, let-7e, miR-140, miR-199a-3p, and miR-320a reached their highest levels in the present study (Table 3). Remarkably, the expression levels of the specific expressed miRNAs were lower than the majority of co-expressed miRNAs.

Table 3. Highly expressed miRNAs (top 10) in the Qira black sheep and Hetian sheep libraries.

Qira black sheep		Hetian sheep	
miRNA	Count	miRNA	Count
miR-140	160,935	miR-103	66,097
miR-145	113,708	miR-107	89,890
miR-199a-3p	117,711	miR-140	155,096
miR-29a	110,856	miR-199a-3p	139,596
miR-320a	106,567	miR-320a	107,444
let-7a	1,541,694	let-7a	1,712,053
let-7b	1,432,265	let-7b	1,854,932
let-7f	1,276,451	let-7c	1,029,291
let-7c	1,219,782	let-7e	145,763
let-7e	122,271	let-7f	1,368,500

Differential expression analysis of conserved miRNAs

The main aim of this study was to identify miRNAs that may play regulatory roles in sheep reproduction by comparing their expression patterns between Qira black sheep and Hetian sheep ovarian tissue. We examined known miRNAs in the two libraries representing these two breeds. The expression of known miRNAs in the two libraries was demonstrated by plotting a log2-ratio plot and a scatter plot (Figure 2), and the expression profiles between the two libraries are shown in **Table S2**. Analysis of the sequencing results demonstrated that most expression levels were equivalent between the two breeds, but there were also 125 unique miRNA expression differences between the two libraries. Of these 125 unique miRNAs, 101 and 24 unique miRNAs were down- and upregulated in the Hetian sheep library, respectively, compared to the Qira black sheep library. In the Qira black sheep library, of the 10 most highly expressed miRNAs (significantly differentially expressed miRNAs), 9 miRNAs (miR-1, miR-10b, miR-101, miR-125b, miR-145, miR-26a, miR-29c, miR-424-5p, and miR-99a) were downregulated and 1 miRNA (miR-423-5p) was upregulated in the Hetian sheep library; and

of the 10 most highly expressed miRNAs in the Hetian sheep library, 3 miRNAs (miR-423-5p, miR-432, and miR-503-5p) were downregulated and 7 miRNAs (miR-1, miR-10b, miR-101, miR-125b, miR-26a, miR-34c, and miR-99a) were upregulated in the Qira black sheep.

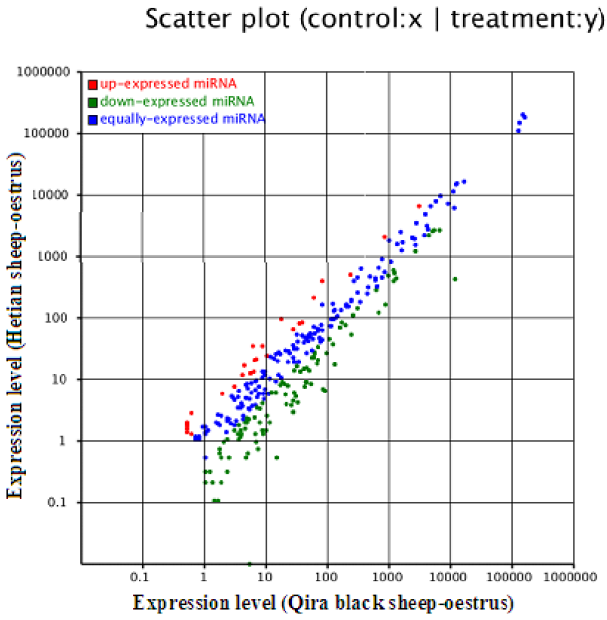

Figure 2. miRNAs differentially expressed in the Qira black sheep and Hetian sheep ovary libraries. Each point represents a miRNA. The X and Y axes show the expression level of miRNAs in the two samples, respectively. And the red points, blue points, and green points represent miRNAs with a ratio >2, 1/2 < ratio ≤ 2, and ratio ≤1/2, respectively. Ratio = normalized expression in treatment / normalized expression in control.

Identification of novel miRNAs

Using the Mireap software (http://sourceforge.net/projects/mireap/), we predicted putative miRNAs by exploring their secondary structure, the Dicer cleavage site, energy, and other features (Bentwich, 2005). Based on Solexa sequencing, 98 putative miRNAs were identified (**Table S3**). Sixty one and 67 novel miRNAs were identified in the Qira black sheep and Hetian sheep libraries, respectively, of which 30 overlapped in both libraries. In addition, the length of the novel miRNA sequences ranged from 20 to 24 nt, with a distribution peak at 22 nt and their 5' ends were comprised most frequently of uridine. These novel sheep miRNAs were not analyzed further, as their expression levels were too low in the two small RNA libraries.

Confirmation of differentially expressed miRNAs

Four differentially expressed miRNAs (miR-23b, miR-26a, miR-432, and miR-760-3p) were analyzed using qPCR to verify the Solexa sequencing data, and the results were consistent with the Solexa sequencing results, showing a similar trend of expression in the two breeds (Figure 3).

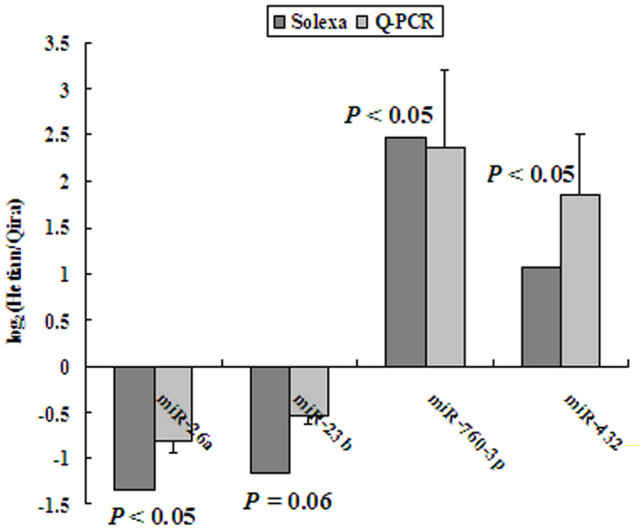

Figure 3. qPCR validation of miRNAs identified in sheep ovaries using Solexa sequencing technology. Log2-ratio >0, and Log2-ratio <0 indicates up- and downregulation in Hetian sheep library compared to Qira black sheep library, respectively. The significance of differences for the expression between Qira black sheep and Hetian sheep was analyzed using a *t*-test, and $P \leq 0.05$ indicates a statistically significant difference.

miRNA target prediction and KEGG Pathway analysis

In this study, the Mireap software was used to predict target genes of the miRNAs. For 395 conserved miRNAs in Hetian sheep and 397 conserved miRNAs in Qira black sheep, 696,823 target sites in 18,645 target genes and 713,562 target sites in 18,645 target genes were predicted, respectively. In addition, 222,765 target sites in 18,606 target genes were predicted for 125 conserved miRNAs that were differentially expressed.

To understand the primary functions of the differentially expressed miRNAs in the two libraries, the target genes of differentially expressed miRNAs were classified according to KEGG functional annotations. KEGG Pathway results showed that 8251 target genes for differentially expressed conserved miRNAs were annotated for 306 biological functions (**Table S4**). The most common pathway was related to metabolism (13.42%). Interestingly, approximately 1.0, 1.6, 0.9, 0.9, and 2.3% of the genes belonged to the TGFβ, Wnt, VEGF, GnRH, and MAPK signaling pathways, respectively, which are known to be closely involved in mammalian reproduction, including follicular development and ovulation.

DISCUSSION

Sheep are an important domestic animal in Xinjiang Province in northwest China. Genetic resources for the high fecundity Qira black sheep and the low fecundity Hetian sheep were created successfully by our research group. The difference in fertility of Qira black sheep and Hetian sheep may have an effect on ovarian function or ovulation rate (Bai, 2007). The miRBase 20.0 database includes 24,521 precursors and 30,424 mature miRNAs from 206 species, but only 105 precursors and 153 mature miRNAs were relevant to sheep; so we compared

our sequences with the known sheep, cattle, and other mammal sequences. In this study, 433 conserved miRNAs were identified in the sheep ovaries (**Table S1**), and this is the first study to characterize genome-wide miRNA profiles between different sheep breeds. Four differentially expressed miRNAs were analyzed using qPCR to verify the reliability of the Solexa sequencing results. Furthermore, we predicted 98 putative miRNAs (**Table S3**); however, the expression levels of most of these miRNAs in the libraries were very low. Nevertheless, these data add new information to existing data on sheep miRNAs. Similarly, genome-wide miRNA expression profiles have also been examined in whole ovaries from mice (Ro et al., 2007; Mishima et al., 2008), cattle (Hossain et al., 2009; Tripurani et al., 2010; Huang et al., 2011), pig (Li et al., 2011), goat (Zhang et al., 2013), and fetal sheep (Torley et al., 2011); however comparisons across different follicular stages, different stages of the estrous cycle or different breeds were not performed (Hossain et al., 2012b; McBride et al., 2012). Recent studies have also proposed roles for miRNAs in the regulation of follicular development and apoptosis (Baley and Li, 2012; Donadeu et al., 2012). With the aim of identifying miRNA populations specifically associated with follicular differentiation, McBride et al. (2012) sequenced small RNA libraries generated from sheep ovarian tissues at specific stages of the ovine estrous cycle representing healthy growing follicles, pre-ovulatory follicles, early corpora lutea, and late corpora lutea, and yielded a total of 212 miRNA sequences in sheep follicles and corpora lutea, including 23 novel miRNAs. In another study, Kim et al. (2010) showed that oxidative stress and hormones may have an influence on the expression of miRNAs during *in vitro* follicular maturation.

To understand whether the two breeds of sheep with different fecundity have different profiles of miRNA expression in ovarian tissue, we identified differentially expressed miRNAs in the ovary tissues of Qira black sheep and Hetian sheep. These results will help to analyze the relationship between sheep miRNAs and reproductive traits, such as follicular development and ovulation rate. A total of 125 conserved miRNAs were significantly differentially expressed in the Qira black sheep and Hetian sheep libraries (**Table S2**). By promoting the degradation of their target mRNAs or repressing translation, miRNAs usually regulate protein expression (Wienholds and Plasterk, 2005). The results of this paper indicate that some miRNAs upregulated in the ovaries of Hetian sheep may inhibit the expression of target genes, which are associated with follicular development or ovulation. On the contrary, some other miRNAs upregulated in the ovaries of Qira black sheep may relieve the repression of target genes, which are associated with follicular development or ovulation; thereby promoting ovulation. For example, many members of the TGFβ superfamily have been shown to be essential for regulating the development of ovarian follicles and, thus fertility, such as affecting oocyte maturation and granulosa cell function. miR-224 was suggested to mediate the stimulatory effects of TGFβ1 on granulosa cell proliferation, aromatase (CYP19A1) expression, and oestradiol release by targeting Smad4 (Yao et al., 2010a). In our study, the expression of miR-224 was significantly upregulated in Qira black sheep compared to Hetian sheep, indicating that miR-224 may have an indirect stimulatory effect on ovarian follicular development in Qira black sheep.

Regardless of species, let-7 family, miR-125b, miR-143, miR-145, and miR-199b, miR-21 and miR-99a have been found to be the most commonly abundant miRNA populations within ovaries (Hossain et al., 2009; Tripurani et al., 2010; Huang et al., 2011; Li et al., 2011; Torley et al., 2011; Zhang et al., 2013). The present study also showed that these miRNAs were expressed at high levels in both libraries, and miR-125b, miR-145, and miR-99a were significantly upregulated in Qira black sheep compared to Hetian sheep. Interestingly, several well-known target genes of miR-125b (IRS1, NR5A2, PTGS2, TGFβR3, etc.), miR-145

(ActRIB, BMP4, CDC2, NOBOX, IGFBP2, IRS1, VEGFA, etc.), and miR-99a (EDF1, IGFBP2, etc.) were predicted in this study, and these have already been validated and reported as ovarian function-related genes (Hossain et al., 2009; Hossain et al., 2012b; Yan et al., 2012). One previous study has demonstrated that miR-125b, miR-145, and miR-99a were expressed at significantly higher levels in the ovaries of pregnant goats compared to non-pregnant goats (Zhang et al., 2013). In addition, miR-125b, miR-145, and miR-99a were expressed at higher levels in sheep follicular tissues (representing healthy growing and pre-ovulatory follicles) compared to luteal tissues (representing early corpora and late corpora lutea) (McBride et al., 2012). *In vitro*, the expression of miR-125b was upregulated in granulosa cells after 12 h of FSH exposure (Yao et al., 2010b). Similarly, another study reported that treatment of cultured bovine granulosa cells with forskolin (an adenylate cyclase agonist) to promote luteinisation was followed by a decrease in the levels of miR-125b and miR-145 (McBride et al., 2012). Additionally, Yan et al. (2012) reported attenuation of activin induced proliferation of mouse granulosa cells by miR-145 targeting ActRIB. Therefore, miR-125b, miR-145, and miR-99a may play a specific role in the regulation of ovarian function and mammalian reproduction.

In contrast to miR-125b, miR-145, and miR-99a, miR-503-5p was significantly upregulated (1.25-fold) in Hetian sheep compared to Qira black sheep. In previous studies, miR-503 has been found to be involved in the regulation of follicular development (Lei et al., 2010; McBride et al., 2012), and the ovary is the main site of miR-503 expression in the body (Hossain et al., 2009; Lei et al., 2010; McBride et al., 2012). Target gene prediction studies have indicated that miR-503 may bind to the IGF1R, Wnt4, Wnt3A, and ActR genes, which may regulate the development of ovarian follicles, and the function of granulosa cells and luteal cells (Hsieh et al., 2002; Lei et al., 2010). Moreover, a previous study on mice reported that ovarian miR-503 levels decreased in response to treatment with eCG and hCG, and overexpression of miR-503 in granulosa cells resulted in significant downregulation of different transcripts (ActR, Bcl2, Ccnd2, INHA, CYP19A1, and Cdkn1b) involved in cell proliferation and differentiation (Lei et al., 2010). Consistent with these results, during the sheep oestrous cycle, miR-503 levels transiently decreased in sheep pre-ovulatory follicles followed by recovery in corpora lutea (McBride et al., 2012). Taken together, the above findings are consistent with the notion that miR-503 is involved in regulating mammalian follicle development and ovarian function.

However, it is a challenge to verify the complex functions of miRNAs. Several studies have indicated that specific members of the let-7 family can affect mammalian reproduction, development, cell proliferation, and apoptosis (Roush and Slack, 2008; Miles et al., 2012; Zhang et al., 2013), and bioinformatic analysis indicated that let-7b may bind to the ActRI and Smad2/3 genes of the TGFβ signaling pathway, and may affect follicular development and estrogen secretion (Zhang et al., 2013). In the present study, 6 members of the let-7 family (let-7a, let-7b, let-7c, let-7f, let-7e, and let-7g) were expressed at high levels in both libraries, but the expression levels of these miRNAs were equivalent in Qira black sheep and Hetian sheep ovaries (**Table S2**), which differs from the previous study. These discrepancies will need to be resolved, although they may reflect genuine species differences. Furthermore, in addition to identification and differential expression studies, functional characterization of these miRNAs in sheep follicular development and ovulation, hormone secretion, specific cell lines of ovary at different follicular stages or at different stages of the estrous cycle remain to be explored further (Hossain et al., 2012b), which will further the understanding of the relationship between miRNAs and mammalian reproduction.

In summary, the aim of our study was to examine different ovarian profiles of miRNA

in sheep and to evaluate miRNA functions in the regulation of sheep reproduction through the identification of differentially expressed miRNAs in Qira black sheep and Hetian sheep ovaries. In this study, 433 conserved and 98 potential novel miRNAs were detected, and 125 conserved miRNAs were significantly differentially expressed in Qira black sheep and Hetian sheep ovarian libraries. The information generated from this study will enrich the sheep miRNA databases and provide insights into sheep ovarian miRNAs, which can be further characterized for their role in ovulation and female fertility.

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Supplementary material

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