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SHORT COMMUNICATION

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Identification and characterization of miRNAs in the ovaries of a highly prolific sheep breed

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Summary

Until recently, there have been few studies concerning miRNAs or miRNA-mediated biological processes in sheep (*Ovis aries*). In the present study, we used a deep-sequencing approach to examine ovarian miRNAs and the mRNA transcriptomes in two ewes of a highly prolific breed, Finnsheep. We identified 113 known sheep miRNAs, 131 miRNAs conserved in other mammals and 60 novel miRNAs, the expression levels of which accounted for 78.22%, 21.73% and 0.05% of the total respectively. Furthermore, the 10 most abundantly expressed miRNAs in the two libraries were characterized in detail, and the putative target genes of these miRNAs were annotated using GO annotation and KEGG pathway enrichment analyses. Among the target genes, intracellular transducers (*SMAD1*, *SMAD4*, *SMAD5* and *SMAD9*) and bone morphogenetic protein (BMP) receptors (*BMPR1B* and *BMPR2*) were involved in the transforming growth factor β (TGFβ) signaling pathway in the reproductive axis, and the most significant GO terms were intracellular part (GO:0044424), binding (GO:0005488) and biological_process (GO:0008150) for cellular component, molecular function and biological process respectively. Thus, these results expanded the sheep miRNA database and provided additional information on the prolificacy trait regulated through specific miRNAs in sheep and other mammals.

Keywords deep-sequencing, mRNA, ewes, prolificacy

Research on microRNAs (miRNAs) as pivotal dysregulation molecules in cell differentiation, apoptosis and metabolism (e.g., the review in De Tullio *et al.* 2014) has increased in popularity. The ovary plays a critical role in female reproduction (e.g., Chen *et al.* 2012). Previous studies have identified novel or specific miRNAs associated with reproduction processes, such as folliculogenesis (Ro *et al.* 2007), corpus luteum insufficiency, infertility (Otsuka *et al.* 2008) and ovulation rates (Carletti *et al.* 2010), in a variety of organisms. However, there are only a few studies on the post-transcriptional regulation of miRNA expression in sheep (e.g., Donadeu *et al.* 2012; McBride *et al.* 2012), reflecting the limited genomic resources available for this species. In the present study, we profiled the miRNA expression patterns in the ovaries of two Finnsheep ewes, a highly prolific breed (e.g., Li *et al.* 2009), using a deep-sequencing approach. We further conducted an integrated investigation of the mRNA and miRNA expression patterns in these samples and identified the target genes of the 10 most abundantly expressed miRNAs and their biological pathways associated with prolificacy.

All the procedures and experimental operations involving animals were performed according to the Finnish laws and EU directives regarding animal experimentation (Decision No. ESAVI/3853/04.10.03/2011 by the National Animal Experiment Board in Finland). The two Finnsheep ewes (17 months old; designated as S48 and S50) were born on the same farm located in Western Finland. These animals were closely related (co-ancestry = 0.375). The ovaries from each of the two ewes were harvested after

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Correction added on 28 December 2015 after first online publication; The author name "Ismael Zhaboyev" should be written as "Ismail Zhaboyev".

slaughter. The ovaries were dissected into four sections and immediately stored in RNAlater (Qiagen) at −20 °C until further use. Total RNA was extracted from the cortex of the ovary using the RNeasy Plus Mini Kit (Qiagen), following the manufacturer's instructions. The RNA concentration and quality were quantified based on the minimal ratio of OD260/OD280 = 1.8 using a spectrometer (Nanodrop). The cDNA libraries were prepared and sequenced on an Illumina HiSeq 2000 platform.

After performing initial quality control checks on raw sequencing data using FASTQC v0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), the adapter sequences were removed using CUTADAPT v1.2.1 (Martin 2011). PRINSEQ-LITE v0.20.4 (Schmieder & Edwards 2011) was subsequently used for quality control and preprocessing of the reads (phred score > 30). The clean reads were compared with ncRNAs (miRNAs, tRNAs, snoRNAs, rRNAs, etc.) deposited in the Rfam v11.0 database for annotation. Clean reads between 18 and 26 nucleotides (nt) in length were used for the downstream analysis.

Reads obtained after filtering were initially mapped on the sheep genome assembly Oar v3.1 (ftp://ftp.ensembl.org/pub/release-74/fasta/ovis_aries/dna/) using the program BOWTIE v2.2.1, tolerating one mismatch and removing reads that mapped to more than five locations in the genome. The mapped reads and the ~70 nt flanking sequences were subsequently processed to predict putative precursor miRNA sequences using the MIRDEEP2 v0.0.5 software package (Friedlaender *et al.* 2012), and the sequences were retained to examine corresponding miRNA expression. The expression of each miRNA was further normalized as actual miRNA count/total count of clean reads × 1 000 000 (Wu *et al.* 2014). Putative precursor miRNAs with 10 or fewer reads and MIRDEEP2 scores lower than 5 were removed. The miRNAs which have been mapped in the database of sheep miRNAs (miRBase database v20.0: http://www.mirbase.org) were denoted as sheep miRNAs, whereas the miRNAs matching with other mammals with fewer than four mismatches were considered as conserved miRNAs. The remaining miRNA molecules were denoted as novel miRNAs hereafter.

Family classifications of the miRNA hairpin sequences in miRBase v20.0 were used to cluster known and conserved miRNAs expressed in the two libraries. We identified the miRNA target genes using the TARGETSCAN algorithms v6.2 (http://www.targetscan.org/), which predicted the 5′ seed regions (2–8 nt) of miRNAs complimentary to the conserved 8- and 7-mer sites in the 3′ untranslated region (3′-UTR) of mRNA (Lewis *et al.* 2005). All expressed genes detected through mRNA-seq in the same samples (S48 and S50; Pokharel *et al.* 2015) were selected as the target database. In addition, gene ontology (GO) and pathway enrichment analyses were applied to these putative miRNA target genes using the GO (http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp) databases respectively. The GO categories with P-value < 0.05 were considered as significantly enriched.

For miRNA validation of eight selected miRNAs (i.e., 26f, 26i, 27f, 27i, 29f, 29i, 30f and 30i; see Table S2), quantitative reverse transcription PCR (qRT-PCR) was performed using the miScript SYBR® Green PCR Kit with primer assays (Qiagen). The PCR volume was prepared using 12.5 µl of 2× QuantiTect SYBR Green PCR master mix, 2.5 µl of 10× miScript universal primer, 2.5 µl of 10× primer assay set and 5 µl of ddH_2O, then added to 2.5 µl of cDNA template. The reactions were incubated at 95 °C for 15 min followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s. All the reactions were run in triplicate with U6 as an internal control. The relative quantification of miRNA expression was calculated using a comparative threshold cycle (Ct) method as described in Abd El Naby *et al.* (2013), and results were reported as the relative expression or n-fold difference to the calibrator (control group) after normalization of the transcript amount relative to the value of the endogenous control (U6). All the data were analyzed using the SAS software package for Windows (release 8.02, SAS Institute Inc.). Data of three replicates of each treatment were analyzed with one-way ANOVA followed by the t-test (mean ± SE) using the general linear model (GLM) procedure.

We obtained a total of 33 692 841 and 56 545 915 raw reads for the S48 and S50 libraries respectively. After filtering, 30 649 456 reads in S48 and 50 103 765 reads in S50 were retained as high-quality reads. Moreover, we obtained 21 661 491 (69.35%) and 24 835 407 (48.62%) reads of 18–26 nt in length, including 592 784 and 588 289 unique reads for the S48 and S50 libraries respectively. Among the reads, 19 766 731 (91.25%) reads for S48 and 21 429 822 (86.29%) reads for S50 were mapped to the sheep genome (Table 1, Fig. S1). We also detected other types of small RNAs, such as tRNAs and snoRNAs, after blasting against the Rfam database (Table 1, Fig. S2), thereby providing a resource for further studies on the prolificacy trait in sheep.

In total, we identified 304 mature miRNAs, including 113 known miRNAs in sheep, 131 miRNAs conserved in other mammals and 60 novel miRNAs (Table S1). All the novel miRNAs identified here are deposited in GenBank (Accession nos. KT891352–KT891411. The miRNA expression analysis indicated that sheep miRNAs were dominant (78.22%, 27 876 700.21/35 640 883.14), followed by conserved miRNAs (21.73%, 7 744 401.16/35 640 883.14) and novel miRNAs (0.05%, 19 781.78/35 640 883.14) (Fig. 1a). We compared miRNA expressions between S48 and S50 libraries, which showed a significant correlation between each other ($R^2 = 0.8056$, P-value < 0.001, Fig. 1b). The 10 most abundantly expressed miRNAs (miR-10b, miR-181a, miR-26a, miR-

Category	S48	S50	Total
Raw reads	33 692 841	56 545 915	90 238 756
After removing adaptor and low-quality reads	30 649 456	50 103 765	80 753 221
miRNA	18 495 469	19 627 408	38 122 877
tRNA	3 007 932	13 915 753	16 923 685
snoRNA	2 607 607	4 537 096	7 144 703
rRNA	1 953 366	4 144 533	6 097 899
Other Rfam sRNA	492 659	1 087 252	1 579 911
Unknown	4 092 423	6 791 723	10 884 146
18–26 nt reads	21 661 491	24 835 407	46 496 898
18–26 nt reads mapped to genome	19 766 731	21 429 822	41 196 553
Total identified miRNA expression levels (normalized)	18 510 352.57	17 130 530.57	35 640 883.14
Sheep miRNAs (113)	15 140 769.83	12 735 930.38	27 876 700.21
MiRNAs conserved in other mammals (131)	3 360 544.65	4 383 856.51	7 744 401.16
Novel miRNAs (60)	9038.09	10 743.69	19 781.78

Table 1 Summary information of the sequencing reads and identified miRNAs.

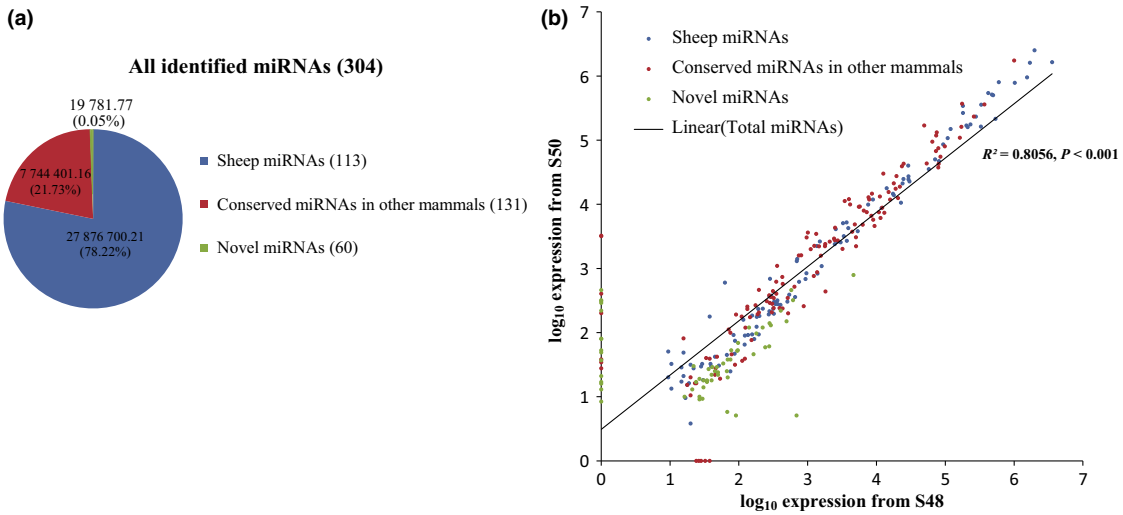

Figure 1 (a) Annotation of the 304 identified miRNAs; (b) comparison of normalized counts from S48 (log_{10} transformed) with normalized counts from S50 (log_{10} transformed). $R^2 = 0.8056$, P-value < 0.001.

143, let-7a, miR-92a, miR-127, miR-125b, let-7f and let-7c) accounted for 68.82% and 67.47% of the total expression in S48 and S50 respectively (Table 2). Nine of these have been previously implicated in sheep mammary gland development (Galio *et al.* 2013), whereas miR-92a has thus far not been identified in sheep. MiR-181a and miR-143 were both shown to inhibit and promote progesterone release in human ovarian cells (Sirotkin *et al.* 2009). In addition, *BMPR1B*, a target of miR-26a, was one of the major determinants for ovulation rate and consequent increase in litter size (Wilson *et al.* 2001). Direct immunofluorescent measurement of granulosa cells surface receptors using flow cytometry demonstrated a significant upregulation of several receptors, among them *BMPR1B* in Booroola Merino sheep with increased ovulation rate (Regan *et al.* 2015). An *et al.* (2015) have reported two single nucleotide polymorphisms (g.151435C>T and g.173057T>C) in the *PRLR* gene which had significant effects on litter size in goats and were regulated by bta-miR-302a. MiR-125b was implicated as an anti-apoptotic regulator to prevent follicular atresia associated with female fertility (Sen *et al.* 2014). Let-7a and miR-125b are two of the five most abundant ovine miRNAs detected at all four stages of follicular and luteal development (McBride *et al.* 2012), also confirming the vital role of miRNAs in reproduction processes. Data presented here are in line of evidence with involvement of miRNAs in prolificacy as genes directly related with trait are targeted by miRNAs detected in the present study.

Ten of the 60 novel miRNAs were expressed in up to 100 reads (except novel-9 in S50) in both libraries (Table 2, Fig. S3). Under stringent parameters for miRNA prediction, the 10 most abundantly expressed novel miRNAs are also indicated to be sheep specific, but further experimental

Table 2 Summary information of the top 10 abundantly expressed known miRNAs (including sheep and conserved miRNAs) and the top 10 abundantly expressed novel miRNAs in the two libraries (ordered by the S48 library).

MiRNA	Mature sequences (5′–3′)	Ref.	Chr	Strand	S48 counts (normalized)	S50 counts (normalized)
miR-10b	ACCCUGUAGAACCGAAUUUGUG	OAR	2	–	3 569 848.71	1 644 891.64
miR-181a	AACAUUCAACGCUGUCGGUGAGU	OAR	12	–	1 980 685.41	2 519 434.30
miR-26a	UUCAAGUAAUCCAGGAUAGGCU	OAR	3	+	1 697 094.11	1 610 518.54
miR-143	UGAGAUGAAGCACUGUAGCUC	OAR	5	+	1 543 167.30	953 143.61
let-7a	UGAGGUAGUAGGUUGUAUAGUU	OAR	2	–	1 022 970.42	784 326.29
miR-92a	UAUUGCACUUGUCCCGGCCUGU	BTA	10	+	1 001 679.28	1 745 979.78
miR-127	AUCGGAUCCGUCUGAGCUUGGCU	OAR	18	+	599 370.90	802 582.91
let-7f	UGAGGUAGUAGAUUGUAUAGU	OAR	X	+	536 847.09	215 112.66
miR-125b	UCCCUGAGACCCUAACUUGUG	OAR	15	–	495 531.77	502 659.17
let-7c	UGAGGUAGUAGGUUGUAUGGUU	OAR	1	–	476 259.79	513 693.43
novel-1	GCAUUCGUGGUUCAGUGGUAGAAUU	NA	25	+	1421.78	4632.98
novel-2	AUUGGCACGUCUUGGAAUGA	NA	X	–	853.70	578.29
novel-3	UCCCUGUCUUCAAUCCUGUAGU	NA	14	–	691.54	689.16
novel-4	AAGUGGAGCCAGAAACCUUGCCA	NA	20	–	453.00	406.23
novel-5	AAGGCCGAGAGGACUGAGCCCU	NA	1	+	297.12	493.22
novel-6	CCCGGUACUGAGCUGACCCGAG	NA	26	+	257.36	291.53
novel-7	UGUGACUGCUAGAACCGCUCCUGC	NA	5	+	174.71	179.70
novel-8	GCGGCCCGCGGGCUCGGAUGCU	NA	1	+	157.98	140.51
novel-9	UGGAGCCGGAGCUGGUUAGCGG	NA	1	–	134.96	97.50
novel-10	GCUCCCCACCCCCUACCUCCACC	NA	3	+	121.36	276.24

Ref. = Reference species; OAR = *Ovis aries*; BTA = *Bos taurus*; Chr = Chromosome; NA = Not available.

validation is needed. This applies especially to the conserved (12 of 131) and novel miRNAs (35 of 60) found in only one of the libraries (Table S1). Eight miRNAs which were identified in only one sequencing library by computational method were chosen for experimental validation (Fig. 2). The qRT-PCR analysis indicated that all the eight novel miRNAs were expressed in this study. In general, the relatively abundant expressions for the four miRNAs (novel-26-i, novel-27-i, novel-29-i and novel-30-i) in the S48 library and the other four miRNAs (novel-26-f, novel-27-f, novel-29-f and novel-30-f) in the S50 library are congruent between the computational prediction and experimental validation (see Fig. 2, Table S1).

Among the 244 known sheep and conserved miRNAs, 237 miRNAs were clustered into 139 miRNAs families, of which the most abundant family, mir-154, comprised 22 miRNAs, followed by let-7 ($n = 9$) and mir-379 ($n = 9$). However, seven miRNAs (miR-3601, miR-7314, miR-6119, miR-6123, miR-2403, miR-7144 and miR-2312) were not assigned to any family and 100 of the 139 mrRNA families included only a single miRNA (Table S3). The 244 miRNAs were well distributed across the chromosomes, except OAR8 and OAR23, and several miRNAs were mapped on OAR18 ($n = 66$) and OARX ($n = 30$) (Fig. S4).

We identified a total of 8788 target genes for the 304 miRNAs, and a majority of the identified genes ($n = 5662$; 64.43%) corresponded to targets for the 10 most abundantly expressed miRNAs. The GO terms enriched with the most significant clusters were intracellular part (GO:0044424) for cellular component, binding (GO:0005488) for molecular function and biological_process (GO:0008150) for biological process (Fig. 3, Table S4).

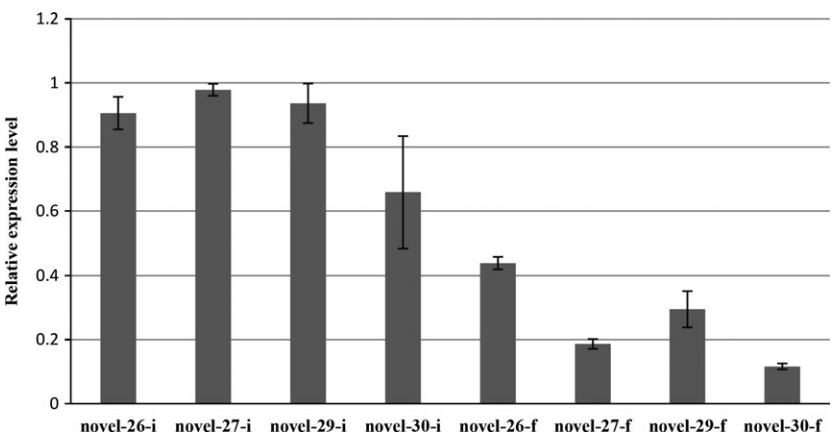

Figure 2 qRT-PCR validation for novel miRNAs identified in the S48 (novel-26-i, novel-27-i, novel-29-i and novel-30-i) and S50 (novel-26-f, novel-27-f, novel-29-f and novel-30-f) libraries. The bars represent mean expression levels ± SE.

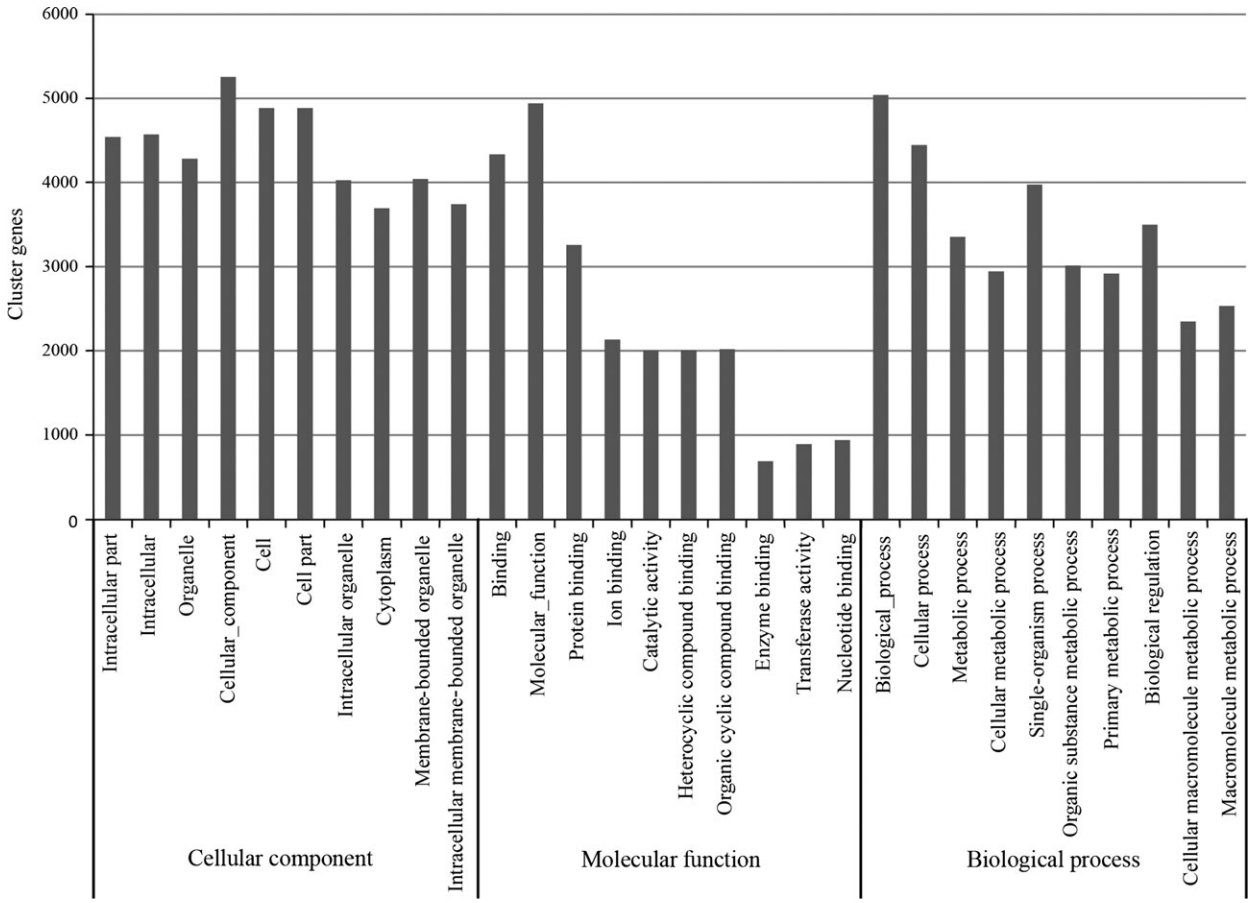

Figure 3 Gene Ontology (GO) annotation of the predicted target genes for the 10 most abundantly expressed miRNAs (the 10 most significantly GO terms for cellular component, molecular function and biological process were listed).

A total of 1300 miRNA target genes were mapped to 300 pathways, including 21 genes (e.g., intracellular transducers, including *SMAD1*, *SMAD4*, *SMAD5* and *SMAD9*) and BMP receptors (*BMPR1B* and *BMPR2*) in the TGFβ signaling pathway (ko04350) in the reproductive axis (Table S5). Thus, these findings provide important insights into the miRNAs associated with the prolificacy trait in sheep. To get more insights in regard to the regulatory role of identified miRNAs, we are going to profile highly abundant miRNAs throughout different stages of estrous cycle and follicular development.

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Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1 Size distribution of the miRNAs in the S48 and S50 libraries.

Figure S2 (a) Reads distribution after removing adaptor and low-quality reads. There were two peaks: one was in 18–26 nt, the standard Dicer processed miRNAs length distribution; the other was 32–34 nt. (b) Total number of clean reads in the S48 library. (c) Total number of clean reads in the S50 library.

Figure S3 Secondary structures of the top 10 novel miRNAs predicted by MIRDEEP2 in the two libraries.

Figure S4 Genomic locations for 244 sheep and conserved miRNAs.

Table S1 The 304 mature miRNAs detected using MIRDEEP2.

Table S2 Primer sequences for eight novel miRNAs.

Table S3 Families for the 237 miRNAs.

Table S4 Significantly enriched GO terms (P-value < 0.05) associated with cellular component, molecular function and biological process for the target genes of the top 10 abundantly expressed miRNAs.

Table S5 KEGG pathways for the target genes of the 10 most abundantly expressed miRNAs.