Differential expression profiles of microRNAs in musk gland of unmated and mated forest musk deer (*Moschus berezovskii*)

Hang Jie[1,*], Zhongxian Xu[2,3,*], Jian Gao[2], Feng Li[2,3], Yinglian Chen[1], Dejun Zeng[1], Guijun Zhao[1] and Diyan Li[2]

[1] Chongqing Institute of Medicinal Plant Cultivation, Bio-resource Research and Utilization joint key laboratory of Sichuan and Chongqing, Nanchuan, Chongqing, China

[2] Sichuan Agricultural University, Institute of Animal Genetics and Breeding, College of Animal Science and Technology, Chengdu, Sichuan, China

[3] China West Normal University, Key Laboratory of Southwest China Wildlife Resources Conservation (Ministry of Education), Nanchong, Sichuan, China

[*] These authors contributed equally to this work.

Corresponding author
Diyan Li, diyanli@sicau.edu.cn

ABSTRACT

Background. The formation of musk is a complex biophysical and biochemical process that change with the rut of male forest musk deer. We have reported that the mating status of male forest musk deer might result to the variations of chemical composition and microbiota of musk and its yields. Critical roles for microRNAs (miRNAs) of multi-tissues were profiled in our previous study; however, the role for miRNAs of the musk gland remains unclear in this species.

Methods. In this study, we used Illumina deep sequencing technology to sequence the small RNA transcriptome of unmated male (UM) and mated male (UM) of Chinese forest musk deer.

Results. We identified 1,652 known miRNAs and 45 novel miRNAs, of which there were 174 differentially expressed miRNAs between UM and MM. chi-miR-21-5p, ipu-miR-99b and bta-miR-26a were up-regulated in UM among the 10 most differentially expressed miRNAs. Functional enrichment of the target genes showed that monosaccharide biosynthetic process, protein targeting, cellular protein catabolic process enriched higher in MM. Meanwhile, structural molecule activity, secretion by cell, regulated exocytosis and circulatory system process enriched more in UM, hinting that the formation of musk in UM was mediated by target genes related to exocytosis. The miRNA-mRNA pairs such as miR-21: *CHD7*, miR143: *HSD17B7*, miR-141/200a: *Noc2* might involve in musk gland development and musk secretion, which need to be verified in future study.

INTRODUCTION

Chinese forest musk deer is an economically important species that is mainly distributed in western China. The musk produced by the male musk gland has a unique perfume

and pharmaceutical characteristics, so is often used as a raw material for perfumery and Asian traditional medicine (*Meng et al., 2012*; *Shrestha, 1998*). However, the musk deer population has drastically declined in recent decades through overhunting, causing it to be listed as an endangered species on the IUCN Red List (*Yang et al., 2003*). Musk deer are seasonal breeders, and the rut of the male musk deer starts in October and extends to February of the following year. Furthermore, the formation of musk is also seasonal, the musk-secreting period is usually during May to July and the maturation of musk is usually in August and September (*Zhang et al., 2015*). The formation of musk is a complex biophysical and biochemical process involving microorganisms. Age, nutrition, and the level of sex hormones of the male musk deer have been reported to affect the musk yield (*Zhang, 1983*; *Chen, Ma & Zhou, 1992*; *Bai et al., 2013*). Additionally, we previously showed that unmated male musk deer (22.79 ± 3.6 g, $n = 5$) secrete more musk than mated males (1.12 ± 0.34 g, $n = 5$) (*Li et al., 2016*). We found 137 differentially expressed genes between unmated and mated sexually mature Chinese forest musk deer in our previous study. The "PPAR signaling pathway", "Calcium signaling pathway", "PI3K-Akt signaling pathway" and "Thyroid hormone synthesis" pathways were more abundant in unmated musk deer (*Jie et al., 2019*). In addition, we found the serum testosterone levels in unmated males were much higher than that of mated males, especially during the vigorous musk secretion period (69.44 ± 3.24 nmol/L in unmated males and 57.24 ± 6.65 nmol/L in mated males, $P < 0.05$, $n = 10$) (our unpublished data). However, the molecular mechanism underlying the relationship between mating status and musk yield is unclear.

MicroRNAs (miRNAs) are noncoding, endogenous small RNAs (approximately 22 nucleotides in length) that regulate gene expression by binding to the 3′ untranslated region (3′ UTR) of mRNAs (*Ambros, 2004*). They are evolutionarily conserved in bilaterian animals although the fact that the current nomenclature conventions make annotation rather subjective in accepting putative new miRNA-related sequences (*Wilczynska & Bushell, 2015*; *Takane et al., 2010*; *Desvignes et al., 2015*). The miRNA-mediated repression of gene expression occurs either through the degradation of target mRNAs or the repression of target mRNA translation (*Yekta, Shih & Bartel, 2004*; *Nilsen, 2007*). In metazoans, most miRNAs negatively regulate protein expression by binding to the 3′ UTRs of target mRNAs with imperfect complementarity, but have no influence on mRNA levels (*Catalanotto, Cogoni & Zardo, 2016*). Increasing evidence has demonstrated that miRNAs exhibit spatiotemporal expression patterns, and that they participate in the regulation of a range of biological processes such as cell proliferation and differentiation, organ development, and tumorigenesis (*Ambros, 2004*; *Hwang & Mendell, 2006*; *Krichevsky et al., 2003*). Recently, they were also shown to be involved in the development of gonads and the secretion of sex hormones in mammals. *Bjork et al. (2010)* reported that miR-18 mediates male germ cell maturation by directly targeting heat shock factor 2 in mouse, while miR-17-92 and miR-106b-25 were shown to regulate spermatogonial differentiation in mice (*Tong et al., 2012*). However, the role of miRNAs in the musk gland of male musk deer, as an accessory sex gland, has not been explored.

In this study, we sequenced the small RNA transcriptomes of the musk gland of unmated and mated musk deer using Illumina high-throughput sequencing. Our results will help to better comprehend the roles of miRNAs in musk gland of forest musk deer.

MATERIALS & METHODS

Collection of musk gland

Forest musk deer raised in Chongqing Institute of Medicinal Plant Cultivation (Chongqing, China, altitude: 678 m). Every individual was housed in a 12 m^2 single living room, there were a big play ground in front of 5 living rooms. The concentrate-to-forage ratio of feed was 1:4. The coarse fodders were the plants harvested from the area nearby, including *Ipomoea batatas*, *Pittos porum* tiny leaves, *Broussonetia papyrifera* tiny leaves, and other light green weeds. The concentrates was concluded 65% corn, 25% full-fat soybean, 6% wheat bran, 0.4% NaCl, 1.5% CaCO$_3$, 0.8% CaHPO$_4$, 0.15% muti-vitamin, 0.15% muti-mineral. *Pittos porum* and *Broussonetia papyrifera* tiny leaves were fed at 08:30 every morning and a mixture of concentrates and other coarse fodders were fed at 17:30 every afternoon. And forest musk deer could drink water freely. HIKVISION video cameras were applied to monitored the mating behavior (*Li et al., 2016*). According to the mating situation records, musk gland tissues were collected from unmated ($n = 1$) and mated ($n = 1$) musk deer at the age of ~4.5 which died from earthquake at April 20th, 2013. (Animals that suffered the earthquake did not die immediately, they died from incurable injuries, during which we prepared all stuffs.) Autopsy was conduct within 5 min after its death, the musk gland was dissected and quickly stored in sterile cryopreservation tubes with RNAlater (Ambion), and immediately frozen with liquid nitrogen before transferred it to -80 °C until total RNA extraction. Experimental procedures were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University (permit number: S20151006) and all efforts were made to minimize suffering.

Small RNA library construction and high-throughput sequencing

Total RNA from each sample was extracted with TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The quantity and integrity of RNA were examined using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA) and 1% agarose gel electrophoresis. Total RNA (~20 μg) from the musk glands of the UM and MM was used to construct library, respectively. Small RNAs of 17–35 nt fractions was enriched from these corresponding libraries of UM and MM using polyacrylamide gel electrophoresis, then ligated to adaptors. Subsequently, the adaptor-ligated small RNAs were reverse transcribed into cDNA and amplified using 15 PCR cycles. Finally, TruSeq® Small RNA Library Prep Kit (Illumina) was used to construct the small RNA libraries, subsequently sequenced by a 50-bp single end reads (SE-50) on the Illumina HiSeq 2000 platform at Sangon Biotech (Shanghai, China). Raw data of Illumina sequencing have been deposited in NCBI with to the SRA (Short Read Archive) accession number: No. SRA548312 (https://www.ncbi.nlm.nih.gov/sra/?term=SRA548312) which contain 2 libraries SRR5378549 and SRR5366118.

Basic analysis of small RNA sequencing data

After high-throughput sequencing, the 3′ adaptor sequences were removed using Cutadapt software (version 1.9.1) (*Martin, 2011*), and truncated reads longer than 35 nt or shorter than 17 nt in length were removed. FASTX-Toolkit software (version 0.0.14) (http://hannonlab.cshl.edu/fastx_toolkit/) was used to filter the remaining reads, discarding those with ≥20% of bases with a Phred score <30 using a Fastq quality filter (−q 30 −p 80). Fastx collapse was then used to remove identical reads. To annotate the small RNAs(tRNA, rRNA, small nuclear (sn)RNA, small nucleolar (sno)RNA, small conditional (sc)RNA, and repeats), remaining reads were compared against tRNAdb (http://trna.bioinf.uni-leipzig.de/) (*Jcohling et al., 2009*), SILVA rRNA (https://www.arb-silva.de/) (*Quast et al., 2013*), GenBank (https://www.ncbi.nlm.nih.gov/genbank/) (*Benson et al., 2013*) and RepBase (http://www.girinst.org/repbase/) (*Bao, Kojima & Kohany, 2015*) databases using Bowtie v.1.0.0 software (*Langmead, 2010*). Because no information exists about Chinese forest musk deer miRNAs, the unannotated reads were compared against known metazoan mature miRNAs obtained from miRBase 21.0 (http://www.mirbase.org/) (*Kozomara & Griffithsjones, 2014*) to identify known miRNAs. Only reads perfectly matched with miRBase were considered as known miRNAs. The unmatched reads were aligned with the integrated transcriptome of musk deer to predict novel miRNAs by miRDeep2 software (*Friedländer et al., 2008*). Only reads whose hairpin structure had a significant randfold *P*-value (<0.05) and miRDeep score higher than 5 could be considered as novel miRNAs.

Differential expression analysis of known miRNAs

To identify known miRNAs that were differentially expressed between unmated and mated musk deer, miRNA reads were normalized using tags per million ((TPM) = Actual miRNA count/Total clean reads count*1,000,000). When the TPM value of a particular miRNA is ≤1 in two groups, this miRNA cannot be used to perform further differential expression analysis. Then, the DEGseq R package (http://www.bioconductor.org/packages/release/bioc/html/DEGseq.html) (*Wang et al., 2010*) was used to calculate fold-change and *P*-value based on normalized expression values. Only miRNAs with fold-changes ≥2 and *P*-values <0.001 were defined as differentially expressed.

Prediction of target genes of differentially expressed miRNAs

MiRanda3.3a (*Enright et al., 2003*) and RNAhybrid2.2.2 (*Krüger & Rehmsmeier, 2006*) software were used to identify putative target genes of differentially expressed miRNAs by aligning them with the reference genes of musk deer annotated in our previous study (*Jie et al., 2019*). In miRanda, putative target genes were filtered with a threshold of >150 and a minimum free energy of <−20 kcal/mol. Human 3′ UTRs were consulted to predict target genes, and RNAhybrid parameters were set as follows: (1) minimum free energy <−25 kcal/mol; and (2) maximum internal loop size of four nucleotides. Genes commonly predicted by both types of software were defined as target genes.

Functional enrichment of putative target genes of the top 10 expressed miRNAs in UM and MM

We selected putative target genes of the 10 most highly expressed miRNAs of UM and MM respectively to perform functional enrichment using metascape with default parameters (*Zhou et al., 2019*). The details were as follows: Initially, we chose "Customer Analysis" to avoid redundancy of multiple annotated databases. We selected "GO Molecular Functions" and "GO Biological Processes" for functional enrichment, with the default parameters of minimum enrichment: 1.5, P valve cutoff: 0.01.

RESULTS

Overview of sequencing data

A total of 26,179,144 and 23,573,546 raw sequences were obtained from unmated ($n = 1$) and mated ($n = 1$) groups, respectively. After removing adaptor sequences, truncating reads longer than 35 nt or less than 17 nt, and low-quality sequences, a total of 19,435,154 and 16,818,354 clean reads (including 1,206,318 and 1,518,258 unique reads) were obtained, respectively (Table S1). The length distribution of the total clean reads in the two libraries was similar (Fig. 1). We found that the majority of small RNAs were 20–24 nt long, and that the most abundant reads were 22 nt, which accounted for 32.82% and 25.65% in unmated and mated libraries respectively, followed by 21 nt (18.61% and 18.21%), and 23 nt (14.52% and 11.40%). Another small peak distribution at 31–32 nt also accounted for a higher proportion in the two libraries.

A total of 5,587,719 (28.75%) and 5,932,267 (35.27%) total reads in the unmated and mated libraries, respectively, were annotated as tRNA, rRNA, snRNA, snoRNA, scRNA, and repeats (Fig. 2, Table S2). Subsequently, the unannotated reads were mapped to the mature metazoan miRNAs in miRBase21.0 to identify known miRNAs in Chinese forest musk deer. A total of 5,512,277 and 3,455,159 reads were mapped to the mature metazoan miRNAs, which accounted for 28.36% and 20.54% of total clean reads in unmated and mated libraries, respectively. We identified 1,459 (distributed in 600 miRNA families) and 1,476 (distributed in 605 miRNA families) known miRNAs in the unmated and mated libraries, respectively (Table S3). Among the known miRNAs, 1,283 conserved miRNAs were present in both libraries and 369 miRNAs were detected in only one sample. Furthermore, we found that the count of reads for known miRNAs has large difference in the expression frequency between two libraries. In the unmated library, eleven miRNAs (bta-miR-26a, gga-miR-21-5p, chi-miR-21-5p, bta-miR-125b, ipu-miR-99b, bta-miR-27b, chi-miR-199b-3p, xtr-miR-26, bta-let-7g, bta-let-7b and chi-miR-29a-3p) were the dominant expressed miRNAs, with more than 100,000 reads (Table S3). However, In the mated library, bta-miR-145, gga-miR-21-5p, oar-miR-143, bta-miR-26a, chi-miR-21-5p and bta-miR-125b were predominately expressed miRNAs, each with more than 100,000 reads (Table S3).

Using the criteria for novel miRNA identification, we identified 45 novel miRNAs in the two libraries (Table S4) that we named "mbe-miR-new-N" (N =1–45). The novel mature miRNAs ranged in length from 20 to 24 nt, while the reads count was 6–937,639 in the two libraries.
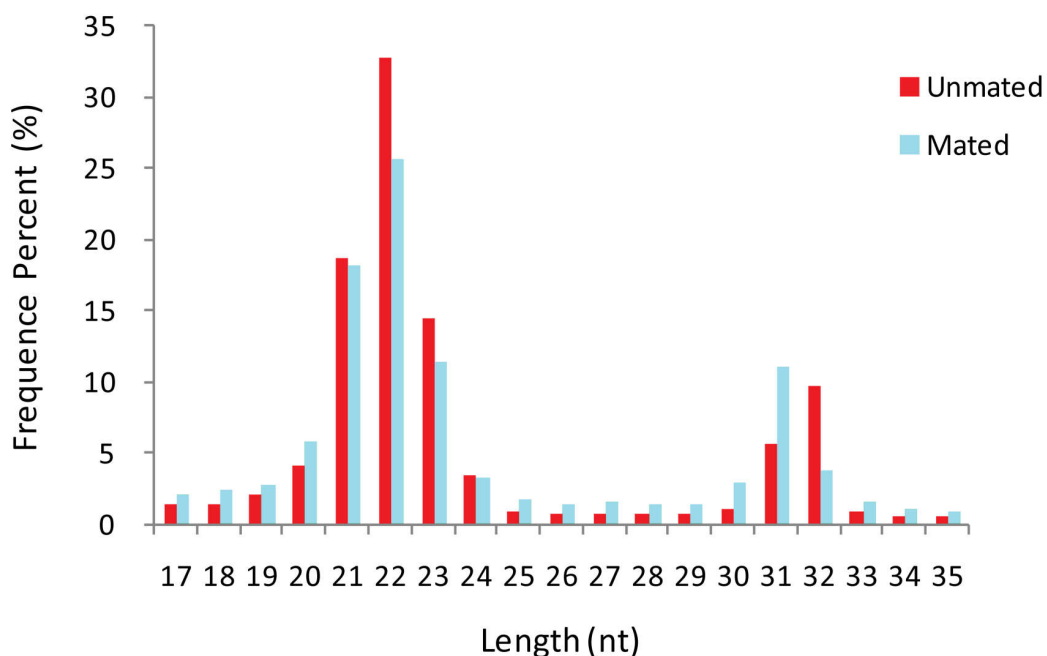
Figure 1 Length distribution-revised.

Differential expression analysis of known miRNAs

To investigate differences in expression levels of known miRNA between unmated and mated individuals, we identified 174 known miRNAs that were differentially expressed between the two groups. Compared with miRNA expression in mated musk deer, 113 miRNAs were significantly down-regulated and 61 were significantly up-regulated in unmated musk deer (Fig. 3 and Table S5). Among the differentially expressed miRNAs, the fold-change of most miRNAs ranged from 1.5 to 4, with mdo-miR-206 having the highest fold-change (-7.49) in the unmated library compared with the mated library (Table S5). Notably, although chi-miR-21-5p, ipu-miR-99b and bta-miR-26a are up-regulated in unmated male, they are highly expressed in both libraries (Table 1).

Target gene prediction and functional enrichment analysis

To understand the role of miRNAs, it is necessary to predict the target genes of differentially expressed miRNAs. We obtained a total of 5,016 putative target genes for 174 differentially expressed miRNAs using miRanda (version 3.3a) and RNAhybrid (version 2.1.2) software. We further predicted the putative target genes of the 10 most expressed differentially expressed miRNAs, 782 and 714 target genes of UM and MM are obtained, respectively. Functional enrichment analysis showed that structure molecule activity, regulated exocytosis, regulation of secretion by cell, circulatory system process and regulation of proteolysis are more enriched in target genes of miRNA in unmated male. Some biological functions such as monosaccharide biosynthetic process, protein targeting, cellular protein catabolic process are more active in mated male (Fig. 4).
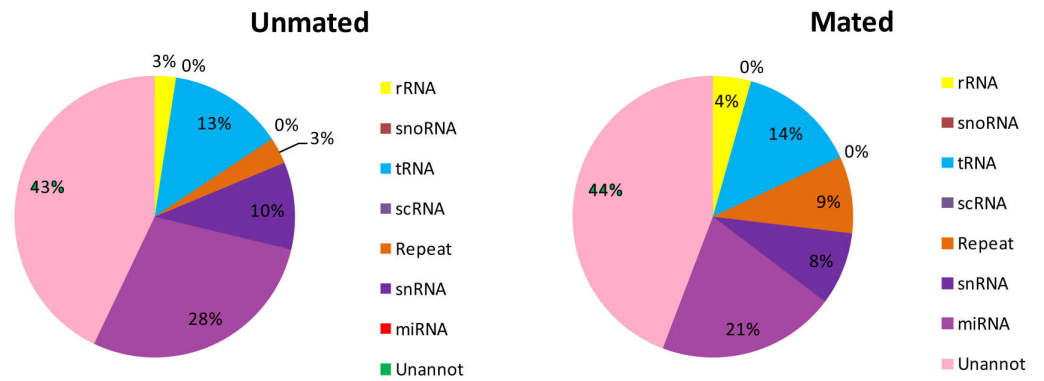
Figure 2 Distribution of small RNAs among different categories in unmated and mated libraries.
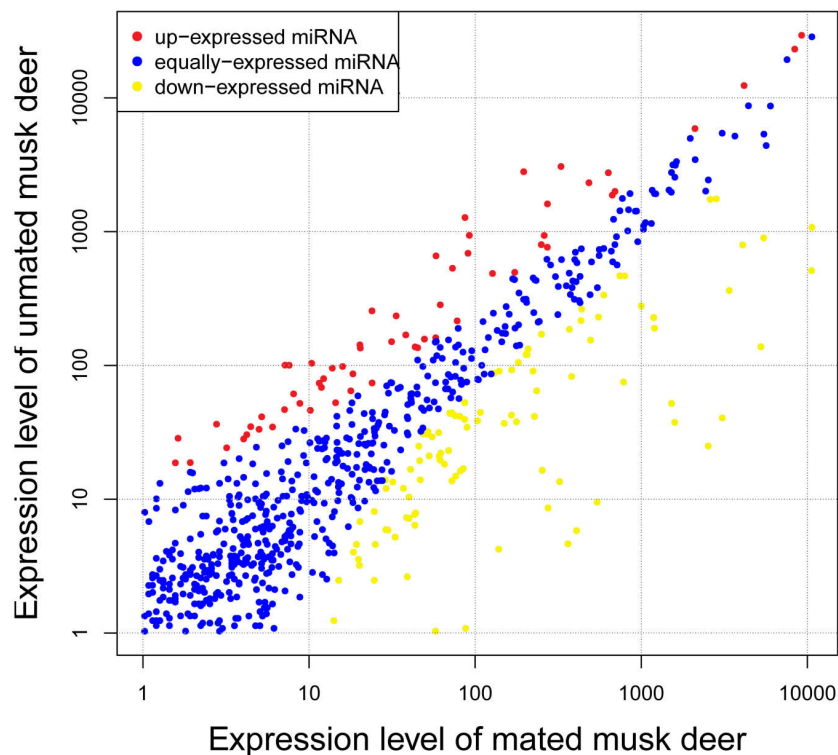
Figure 3 Differential expression profile of known miRNAs in unmated and mated musk deer.

DISCUSSION

Although several studies have investigated the regulation of mammalian reproductive systems by miRNAs, these have mainly concentrated on model animal species and agricultural animals (*Bjork et al., 2010*; *Tong et al., 2012*; *Yao et al., 2014*; *Lannes et al., 2015*). To our knowledge, no research has yet been conducted on the role of miRNAs in musk deer. In the present study, we observed that most musk deer small RNAs were 20–24 nt

Table 1 List of the 10 most differentially expressed miRNAs in unmated and mated males.

microRNAs names	TPM (UM)	TPM (MM)	log2(FC)	Type
bta-miR-26a	29,296.45	9,121.11	1.22	Most differentially expressed in both groups
chi-miR-21-5p	23,064.85	8,289.75	1.01	
ipu-miR-99b	12,327.71	4,106.88	1.12	
xtr-miR-26	5,879.14	2,080.29	1.03	Most differentially expressed in UM
ggo-miR-200b	3,059.92	324.53	2.77	
bta-miR-200c	2,799.67	193.54	3.39	
pma-miR-205a-5p	2,748.37	625.92	1.67	
bta-miR-21-5p	2,311.64	478.29	1.81	
bta-miR-195	1,993.76	685.68	1.07	
oar-miR-27a	1,868.73	660.89	1.03	
bta-miR-145	1,075.01	10,515.24	(3.76)	Most differentially expressed in MM
oar-miR-143	510.42	10,461.13	(4.82)	
ccr-miR-143	896.88	5,387.74	(3.05)	
bta-miR-1	137.38	5,183.03	(5.70)	
xtr-miR-22-3p	793.15	4,025.78	(2.81)	
dre-miR-145-5p	361.56	3,340.22	(3.67)	
xtr-miR-451	40.39	3,036.68	(6.70)	

in size, with 22 nt-length sequences dominating, which is consistent with the typical length of Dicer-processed small RNA products (*Zhang, Stellwag & Pan, 2009*). Interestingly, we also identified a small peak at 31–32 nt which has not been reported in other ruminant animals (*Hossain et al., 2009*; *Ji et al., 2012*). However, a similar phenomenon was seen in the large yellow croaker (*Pseudosciaena crocea*) (*Qi et al., 2014*), in which Qi et al. detected a primary peak at 20–24 nt and a secondary peak at 27–30 nt; this latter peak mainly derived from nuclear tRNA and rRNA. Therefore, it is possible that the 31–32 nt miRNAs in musk deer may also derive from nuclear tRNA and rRNA.

We identified 174 miRNAs are significantly differentially expressed between the unmated and mated males, among which chi-miR-21-5p, ipu-miR-99b and bta-miR-26a are highly expressed in both unmated and mated males. A total of 5,016 putative target genes are obtained, 782 and 714 target genes of UM and MM are predicted, respectively.

We also showed that chi-miR-21-5p was abundant in unmated (448,269 reads) and mated (139,420 reads) musk deer, and that it exhibited significant differential expression between the two groups. Carletti et al. reported that knockdown of miR-21 in ovaries increased granulosa cell apoptosis and decreased the ovulation rate (*Carletti, Fiedler & Christenson, 2010*). Another study showed that hsa-miR-21 expression was regulated by ovarian steroids in mouse granulosa cells and human endometrial stromal cells (*Pan et al., 2007*). In this study, *CHD7* was predicted as the target of miR-21-5p, mutations of this gene cause gonadal endocrine abnormalities, which manifests biochemically as hypogonadotropic hypogonadism (low sex steroid hormone and gonadotropin levels) (*Kim et al., 2008*; *Neocleous et al., 2020*; *Xu et al., 2018*). Furthermore, *CHD7* was enriched
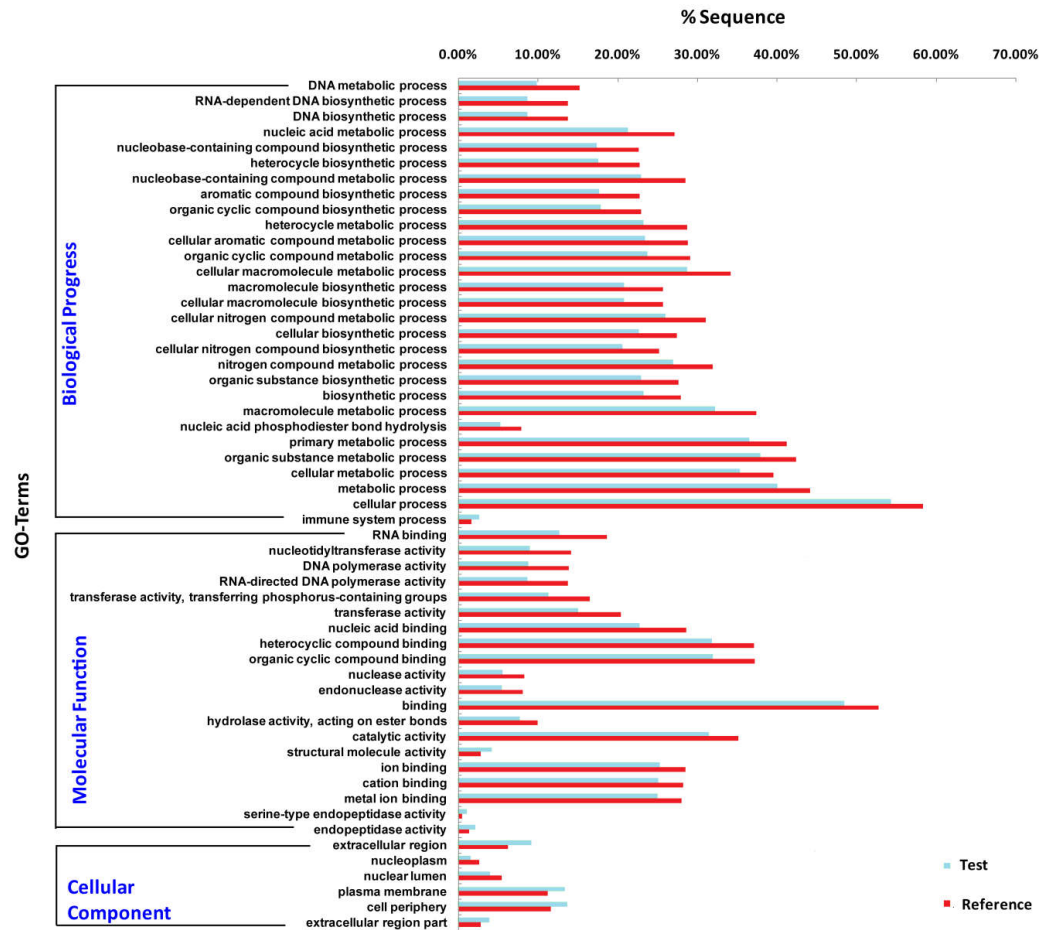
Figure 4 Functional enrichment for the putative target genes of the 10 most differentially expressed miRNAs.

in "circulatory system process" and "regulation of secretion by cell", suggesting that miR-21-5p might be crucial to endocrine function of musk gland in male musk deer.

MiR-143 was previously found to be highly expressed in mammalian gonads, indicating that it plays an important role in the mammalian reproductive system (*Hossain et al., 2009*; *Xu et al., 2018*; *Landgraf et al., 2007*; *Mishima et al., 2008*; *Li et al., 2011*). Its expression was reported to be under the negative control of follicle-stimulating hormone, which regulates folliculogenesis, and to gradually increase during follicular development (*Yao et al., 2009*). We found that miR-143 (including hsa-miR-143-5p, oar-miR-143, bta-miR-143, chi-miR-143-5p, lla-miR-143, and ccr-miR-143) was significantly down-regulated in unmated musk glands compared with mated musk glands. *HSD17B7*, as a target of miR-143, with higher expression level in testis than ovary, encodes sex-steroidogenic enzymes that is essential for testosterone metabolism (*Nyuji et al., 2020*; *Kimoto et al., 2010*). The participation of *HSD17B7* in regulation of secretion by cell testified the putative biological function of miR-143 in testosterone metabolism and musk secretion.

We also showed that the miR-200 family, including bta-miR-141, bta-miR-200a, and bta-miR-200c was significantly up-regulated in unmated musk glands compared with mated musk glands. Previous work suggested that the expression of miR-141, miR-200a, and miR-200c gradually decreased during primordial germ cell development (*Hayashi et al., 2008*), while another study reported significant increases in miR-141 within the seminal plasma of non-obstructive azoospermia patients compared with fertile controls (*Wu et al., 2013*). We predicted *Noc2* as a putative target of miR-141/200a, the encoded dual effector Noc2 has a crucial role in secretory-granule exocytosis both in endocrine and exocrine cells, secretory vesicles exocytosis exerts a variety of biological effects, it involves not only in the control of hormone exocytosis, but also in exocrine secretion including the determination of the size and shape of secretory granules (*Cheviet, Waselle & Regazzi, 2004*; *Masanari et al., 2004*; *Kohichi et al., 2017*). Musk gland's seasonal development and musk secretion are regulated by sex hormones produced by the testis under the control of the hypothalamus-pituitary-testis system (*Zhang et al., 2017*), suggesting a possible involvement of Noc2.

Among the differentially expressed miRNAs, the muscle-specific miRNAs of pma-miR-133b-3p and cgr-miR-1 were identified in musk gland. This phenomenon was consistent with the findings of Huang's study, they also identified miR-133 and miR-1 in the testis and ovary of Holstein Cattle (*Huang et al., 2011*). Intriguingly, these miRNAs have higher expression in mated library compared with unmated library. This result suggested that the previous study, which reported muscle -specific miRNAs, might be have some limits and need to be further verified (*Mishima et al., 2008*).

Functional enrichment provides an understanding of the roles of differentially expressed miRNA target genes. Our results illustrated that more targets were involved in "structure molecule activity", "regulated exocytosis", "regulation of secretion by cell", "circulatory system process" and "regulation of proteolysis" for unmated male. Genes related to "monosaccharide biosynthetic process", "protein targeting", "cellular protein catabolic process" were more active in mated male. Which indicated that the most differentially expressed miRNAs were responsible for musk formation and secretion. Especially the pathway "regulation of secretion by cell" contains hormone secretion, hormone transport, establishment of protein localization to extracellular region, signal release etc. We deduced that miRNAs might be encapsulated by exosomes and secreted by musk gland cells, transported to target organs like testis through circulatory system.

The limited sample ($n = 1$) of each group was a vital shortcoming in our study due to the difficulty of sampling. Actually it was forbidden to sacrifice forest musk deer for sample collection. It is a rare protected animal and listed as an endangered species on the IUCN Red List. Due to the hardship of sample collection technical replicates and further validation experiments are indispensable to confirm our results.

CONCLUSIONS

In summary, this study is the first to systematically characterize miRNA patterns of the musk gland from Chinese forest musk deer. We identified 1,652 known and 45 novel

miRNAs from two libraries of UM and MM. Findings of functional enrichment suggest that miRNAs might affect musk yield in the male musk deer by regulating secretion and transport of hormones, which explains why unmated musk deer secrete more musk than mated musk deer. The putative miRNA-mRNA pairs (miR-21: *CHD7*, miR143: *HSD17B7*, miR-141/200a: *Noc2*) might involve in musk gland development and musk secretion, which need to be verified in our future study. These data will provide a comprehensive reference for further research into the roles of miRNAs in musk glands of musk deer, and will contribute to an understanding of the molecular mechanism of musk secretion.

ACKNOWLEDGEMENTS

We sincerely thanks Chengli Zheng for her help in sampling.

Funding

This research was funded by the National Natural Science Foundation of China (NSFC31672396 and NSFC81973428) and the Natural Science Foundation of Chongqing (cstc2019jcyj-msxmX0024). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
National Natural Science Foundation of China: NSFC31672396, NSFC81973428.
Natural Science Foundation of Chongqing: cstc2019jcyj-msxmX0024.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Hang Jie conceived and designed the experiments, performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Zhongxian Xu conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Jian Gao and Yinglian Chen analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Feng Li analyzed the data, prepared figures and/or tables, and approved the final draft.
- Dejun Zeng and Guijun Zhao performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Diyan Li conceived and designed the experiments, prepared figures and/or tables, and approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

Institutional Animal Care and Use Committee of Sichuan Agricultural University approved the study (permit number: S20151006).

Data Availability

The raw data of Illumina sequencing are available at NCBI SRA: SRR5378549 and SRR5366118.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.12710#supplemental-information.

REFERENCES

Ambros V. 2004. The functions of animal microRNAs. *Nature* **431**:350–355 DOI 10.1038/nature02871.

Bai K, Ren ZJ, Wang YQ, Fei-Ran L, Yong L, Zhu CS, Tang QS, Liu WH, Wang FR. 2013. An association with gonadal hormone level and musk yield in Moschus berezovskii during musk-secreting period. *Chinese Journal of Veterinary Science* **33**:956–962.

Bao W, Kojima KK, Kohany O. 2015. Repbase Update, a database of repetitive elements in eukaryotic genomes. *Mobile DNA* **6**:11 DOI 10.1186/s13100-015-0041-9.

Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. 2013. GenBank. *Nucleic Acids Research* **41**:D36–D42 DOI 10.1093/nar/gks1195.

Bjork JK, Sandqvist A, Elsing AN, Kotaja N, Sistonen L. 2010. miR-18, a member of Oncomir-1, targets heat shock transcription factor 2 in spermatogenesis. *Development* **137**:3177–3184 DOI 10.1242/dev.050955.

Carletti MZ, Fiedler SD, Christenson LK. 2010. MicroRNA 21 blocks apoptosis in mouse periovulatory granulosa cells. *Biology of Reproduction* **83**:286–295 DOI 10.1095/biolreprod.109.081448.

Catalanotto C, Cogoni C, Zardo G. 2016. MicroRNA in control of gene expression: an overview of nuclear functions. *International Journal of Molecular Science* **17**:1712 DOI 10.3390/ijms17101712.

Chen S, Ma L, Zhou Z. 1992. Effects of different levels of energy, crude protein and amino acid diet on musk output of musk deer. *Journal of Southwest University for Nationalities* **18**:91–95.

Cheviet S, Waselle L, Regazzi R. 2004. Noc-king out exocrine and endocrine secretion. *Trends in Cell Biology* **14**:525–528 DOI 10.1016/j.tcb.2004.08.001.

Desvignes T, Batzel P, Berezikov E, Eilbeck K, Eppig JT, McAndrews MS, Singer A, Postlethwait JH. 2015. miRNA nomenclature: a view incorporating genetic origins,

biosynthetic pathways, and sequence variants. *Trends in Genetics* **31**:613–626 DOI 10.1016/j.tig.2015.09.002.

Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS. 2003. MicroRNA targets in Drosophila. *Genome Biology* **5**:R1 (2003) DOI 10.1186/gb-2003-5-1-r1.

Friedländer MR, Chen W, Adamidi C, Maaskola J, Einspanier R, Knespel S, Rajewsky N. 2008. Discovering microRNAs from deep sequencing data using miRDeep. *Nature Biotechnology* **26**:407–415 DOI 10.1038/nbt1394.

Hayashi K, Lopes SMCDS, Kaneda M, Tang F, Hajkova P, Lao K, O'Carroll D, Das PP, Tarakhovsky A, Miska EA. 2008. MicroRNA biogenesis is required for mouse primordial germ cell development and spermatogenesis. *PLOS ONE* **3**:e1738 DOI 10.1371/journal.pone.0001738.

Hossain MM, Ghanem N, Hoelker M, Rings F, Phatsara C, Tholen E, Schellander K, Tesfaye D. 2009. Identification and characterization of miRNAs expressed in the bovine ovary. *BMC Genomics* **10**:443 DOI 10.1186/1471-2164-10-443.

Huang J, Ju Z, Li Q, Hou Q, Wang C, Li J, Li R, Wang L, Sun T, Hang S, Gao Y, Hou M, Zhong J. 2011. Solexa sequencing of novel and differentially expressed microRNAs in testicular and ovarian tissues in Holstein cattle. *International Journal of Biological Sciences* **7**:1016–1026 DOI 10.7150/ijbs.7.1016.

Hwang HW, Mendell JT. 2006. MicroRNAs in cell proliferation, cell death, and tumorigenesis. *British Journal of Cancer* **94**:776–780 DOI 10.1038/sj.bjc.6603023.

Jcohling FM, Mario Hartmann RK, Sprinzl M, Stadler PF, Pcotz J. 2009. tRNAdb 2009: compilation of tRNA sequences and tRNA genes. *Nucleic Acids Research* **37**:D159–D162 DOI 10.1093/nar/gkn772.

Ji Z, Wang G, Xie Z, Wang J, Zhang C, Dong F, Chen C. 2012. Identification of novel and differentially expressed MicroRNAs of dairy goat mammary gland tissues using solexa sequencing and bioinformatics. *PLOS ONE* **7**:e49463 DOI 10.1371/journal.pone.0049463.

Jie H, Xu ZX, Su Y, Lei MY, Zeng DJ, Zhao GJ, Feng XL, Zheng CL, Zhang CL, Liang ZJ, Li DY. 2019. The transcriptome analysis of males musk gland in *Moschus berezovskii* (Artiodactyla: Moschidae). *The European Zoological Journal* **8**:402–412 DOI 10.1080/24750263.2019.1681525.

Kim H, Kurth I, Lan F, Meliciani I, Wenzel W, Eom S, Kang G, Rosenberger G, Tekin M, Ozata M, Bick P, Sherins R, Walker S, Shi Y, Gusella J, Layman L. 2008. Mutations in CHD7, encoding a chromatin-remodeling protein, cause idiopathic hypogonadotropic hypogonadism and kallmann syndrome. *Ameriacn Journal of Human Genetics* **83**:511–519 DOI 10.1016/j.ajhg.2008.09.005.

Kimoto T, Ishii H, Higo S, Hojo Y, Kawato S. 2010. Semicomprehensive analysis of the postnatal age-related changes in the mRNA expression of sex steroidogenic enzymes and sex steroid receptors in the male rat hippocampus. *Endocrinology* **151**:5795–5806 DOI 10.1210/en.2010-0581.

Kohichi M, Masato T, Toshiaki I, Tetsuro I. 2017. Rab2a and *Rab27a* cooperatively regulate the transition from granule maturation to exocytosis through the dual effector Noc2. *Journal of Cell Science* **130**:541–550 DOI 10.1242/jcs.195479.

Kozomara A, Griffithsjones S. 2014. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Research* **42**:68–73 DOI 10.1093/nar/gkt1181.

Krichevsky AM, King KS, Donahue CP, Khrapko K, Kosik KS. 2003. A microRNA array reveals extensive regulation of microRNAs during brain development. *RNA* **9**:1274–1281 DOI 10.1261/rna.5980303.

Krüger J, Rehmsmeier M. 2006. RNAhybrid: microRNA target prediction easy, fast and flexible. *Nucleic Acids Research* **34**:W451–W454 DOI 10.1093/nar/gkl243.

Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, Pfeffer S, Rice A, Kamphorst AO, Landthaler M. 2007. A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* **129**:1401–1414 DOI 10.1016/j.cell.2007.04.040.

Langmead B. 2010. Aligning short sequencing reads with Bowtie. *Current Protocols in Bioinformatics* **Chapter 11**:Unit 11.7 DOI 10.1002/0471250953.bi1107s32.

Lannes J, L'Hôte D, Garrel G, Laverrière JN, Cohen-Tannoudji J, Quérat B. 2015. Rapid communication: a microRNA-132/212 pathway mediates GnRH activation of FSH expression. *Molecular Endocrinology* **29**:364–372 DOI 10.1210/me.2014-1390.

Li D, Chen B, Zhang L, Gaur U, Ma T, Jie H, Zhao G, Wu N, Xu Z, Xu H, Yao Y, Lian T, Fan X, Yang D, Yang M, Zhu Q, Trask J. 2016. The musk chemical composition and microbiota of Chinese forest musk deer males. *Scientific Reports* **6**:18975 DOI 10.1038/srep18975.

Li M, Liu Y, Wang T, Guan J, Luo Z, Chen H, Wang X, Chen L, Ma J, Mu Z. 2011. Repertoire of porcine MicroRNAs in adult ovary and testis by deep sequencing. *International Journal of Biological Sciences* **7**:1045–1055 DOI 10.7150/ijbs.7.1045.

Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *Embnet Journal* **17**:10–12 DOI 10.1089/cmb.2017.0096.

Masanari M, Takashi M, Tadao S, Miho K, Hidehiro S, Junko N, Atsunori S, Haruhiko K, Masaru M, Toshihiko I, Susumu S. 2004. Noc2 is essential in normal regulation of exocytosis in endocrine and exocrine cells. *Proceedings of the National Academy of Sciences of the United States of America* **101**:313–8318 DOI 10.1073/pnas.0306709101.

Meng X, Liu D, Feng J, Meng Z. 2012. Asian medicine: exploitation of wildlife. *Science* **335**:Letters DOI 10.1126/science.335.6073.1168-a.

Mishima T, Takizawa T, Luo SS, Ishibashi O, Kawahigashi Y, Mizuguchi Y, Ishikawa T, Mori M, Kanda T, Goto T. 2008. MicroRNA (miRNA) cloning analysis reveals sex differences in miRNA expression profiles between adult mouse testis and ovary. *Reproduction* **136**:811–822 DOI 10.1530/REP-08-0349.

Neocleous V, Fanis P, Toumba M, Tanteles G, Schiza M, Cinarli F, Nicolaides N, Oulas A, Spyrou G, Mantzoros C, Vlachakis D, Skordis N, Phylactou L. 2020. GnRH deficient patients with congenital hypogonadotropic hypogonadism: novel genetic findings in *ANOS1*, RNF216, WDR11, FGFR1, *CHD7*, and *POLR3A* genes in a case series and review of the literature. *Frontiers in Endocrinology* **11**:626 DOI 10.3389/fendo.2020.00626.

Nilsen TW. 2007. Mechanisms of microRNA-mediated gene regulation in animal cells. *Trends in Genetics* **23**:243–249 DOI 10.1016/j.tig.2007.02.011.

Nyuji M, Hongo Y, Yoneda M, Nakamura M. 2020. Transcriptome characterization of BPG axis and expression profiles of ovarian steroidogenesis-related genes in the Japanese sardine. *BMC Genomics* **21**:668 DOI 10.1186/s12864-020-07080-1.

Pan Q, Luo X, Toloubeydokhti T, Chegini N. 2007. The expression profile of micro-RNA in endometrium and endometriosis and the influence of ovarian steroids on their expression. *Molecular Human Reproduction* **13**:797–806 DOI 10.1093/molehr/gam063.

Qi P, Guo B, Zhu A, Wu C, Liu C. 2014. Identification and comparative analysis of the Pseudosciaena crocea microRNA transcriptome response to poly(I:C) infection using a deep sequencing approach. *Fish & Shellfish Immunology* **39**:483–491 DOI 10.1016/j.fsi.2014.06.009.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* **41**:D590 DOI 10.1093/nar/gks1219.

Shrestha MN. 1998. Animal welfare in the musk deer. *Applied Animal Behaviour Science* **59**:245–250 DOI 10.1016/S0168-1591(98)00139-7.

Takane K, Fujishima K, Watanabe Y, Sato A, Saito N, Tomita M, Kanai A. 2010. Computational prediction and experimental validation of evolutionarily conserved microRNA target genes in bilaterian animals. *BMC Genomics* **11**:101 DOI 10.1186/1471-2164-11-101.

Tong MH, Mitchell DA, McGowan SD, Evanoff R, Griswold MD. 2012. Two miRNA clusters, Mir-17-92 (Mirc1) and Mir-106b-25 (Mirc3), are involved in the regulation of spermatogonial differentiation in mice. *Biology of Reproduction* **86**:1–10 DOI 10.1095/biolreprod.111.096313.

Wang L, Feng Z, Wang X, Wang X, Zhang X. 2010. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. *Bioinformatics* **26**:136–138 DOI 10.1093/bioinformatics/btp612.

Wilczynska A, Bushell M. 2015. The complexity of miRNA-mediated repression. *Cell Death and Differentiation* **22**:22–33 DOI 10.1038/cdd.2014.112.

Wu W, Qin Y, Li Z, Dong J, Dai J, Lu C, Guo X, Zhao Y, Zhu Y, Zhang W, Hang B, Sha J, Shen H, Xia Y, Hu Z, Wang X. 2013. Genome-wide microRNA expression profiling in idiopathic non-obstructive azoospermia: significant up-regulation of miR-141, miR-429 and miR-7-1-3p. *Human Reproduction* **28**:1827–1836 DOI 10.1093/humrep/det099.

Xu C, Cassatella D, Van Der Sloot A, Quinton R, Hauschild M, De Geyter C, Flück C, Feller K, Bartholdi D, Nemeth A, Halperin I, Djurdjevic S, Maeder P, Papadakis G, Dwyer A, Marino L, Favre L, Pignatelli D, Niederländer N, Acierno J, Pitteloud N. 2018. Evaluating CHARGE syndrome in congenital hypogonadotropic hypogonadism patients harboring *CHD7* variants. *Genetics in Medcine* **20**:872–881 DOI 10.1038/gim.2017.197.

Yang Q, Meng X, Lin X, Feng Z. 2003. Conservation status and causes of decline of musk deer (Moschus spp.) in China. *Biological Conservation* **109**:333–342 DOI 10.1016/S0006-3207(02)00159-3.

Yao G, Liang M, Liang N, Yin M, Lü M, Lian J, Wang Y, Sun F. 2014. MicroRNA-224 is involved in the regulation of mouse cumulus expansion by targeting Ptx3. *Molecular & Cellular Endocrinology* **382**:244–253 DOI 10.1016/j.mce.2013.10.014.

Yao N, Lu CL, Zhao JJ, Xia HF, Sun DG, Shi XQ, Wang C, Li D, Cui Y, Ma X. 2009. A network of miRNAs expressed in the ovary are regulated by FSH. *Frontiers in Bioscience* **14**:3239–3245 DOI 10.2741/3447.

Yekta S, Shih IH, Bartel DP. 2004. MicroRNA-directed cleavage of HOXB8 mRNA. *Science* **304**:594–596 DOI 10.1126/science.1097434.

Zhang B. 1983. Musk-deer: their capture, domestication and care according to Chinese experience and methods. *Unasylva* **35**:16–24.

Zhang B, Stellwag EJ, Pan X. 2009. Large-scale genome analysis reveals unique features of microRNAs. *Gene* **443**:100–109 DOI 10.1016/j.gene.2009.04.027.

Zhang T, Peng D, Qi L, Li W, Fan M, Shen J, Yang L, Wang Y, Wang W, Hu X, Cai R, Zhou Y, Wei Y, Zhou J, Yang S, Hu D, Liu S. 2017. Musk gland seasonal development and musk secretion are regulated by the testis in muskrat (*Ondatra zibethicus*). *Biological Research* **50**:10 DOI 10.1186/s40659-017-0116-9.

Zhang ZM, Yang J, Wang JM, Fu WL, Wang ZY, Ye SH, Fu CM. 2015. Changes of serum gonadal hormones levels during musk-secreting period and estrus of *Moschus berezovskii*. *Journal of Chinese Medicinal Materials* **38**:240–244.

Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, Benner C, Chanda SK. 2019. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nature Communications* **10**:1523 DOI 10.1038/s41467-019-09234-6.