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Comparative analysis of miRNAs in exosomes released by sheeppox virus-infected ovine testicular cells

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ABSTRACT

Exosomes, secreted by various cells, are nanometer-scale vesicles with the functions in intercellular communication. To understand a role of exosomal miRNAs in the sheeppox virus infection, exosomes were isolated from sheeppox virus-infected sheep testicular cells 0 h, 24 h and 72 h post infection. The results of transmission electron microscopy and size distribution showed that all three exosome samples were spherical particles with negatively-stained membrane, ranging from 39 nm to 127 nm in diameter. A total of 106 known and 279 novel miRNAs were identified, and 78 known and 54 novel miRNAs were commonly detected in three exosome samples. Compared with the exosomes by the uninfected controls, a total of 34 known miRNAs were aberrantly expressed in the exosomes from infected cells. In agreement with the sequencing data, the expression of oar-miR-21 and oar-miR-10b was shown to be the highest in exosomes at 24 h after SPPV-infected, and the expression of oar-let-7f was the highest in exosomes at 72 h. Conversely, the expression of oar-let-7b and oar-miR-221 was significantly decreased 24 h and 72 h post infection compared with 0 h. The analysis results also revealed that differentially expressed miRNAs were mostly involved in an immune system process and stimulus response. These results provide rich data to further investigate a role of exosomal miRNAs in SPPV-host interactions.

1. Introduction

Sheeppox virus (SPPV), a complex double-stranded DNA virus of the Genus Capripox, can cause sheeppox [1]. The disease is one of serious pox diseases in domestic animals with considerable morbidity, and a mortality rate is 10–50 % in adult sheep and 100% in lambs [2]. In China, the disease is endemic in many regions, such as Xinjiang, Gansu, Guizhou and Yunnan [3]. Sheeppox outbreaks cause considerably economic losses to sheep industry. Sheeppox has been classified as a notifiable animal disease by World Organization for Animal Health (OIE) and is also considered as a potential terrorism biochemical weapon [4]. At present, there are not effective approaches available for the treatment of sheeppox [2]. Therefore, it is very urgent to clarify the mechanisms of pathogenesis, which will provide clues for the development of antiviral reagents.

Exosomes, being 40 nm–130 nm in diameter, are secreted through the endocytic cellular pathway by a cell. These nanosized membrane-wrapped vesicles carry proteins, lipids and RNAs, being involved in cell-to-cell substance transportation and signaling transduction [5]. Numerous studies have reported that virulence factors are released into recipient cells via exosomes and are involved in viral infection [6]. Of virulence factors, miRNAs are involved in a variety of biological pathways at different development stages in different cell types [7], including cellular immunity, apoptosis, signal transduction and cell differentiation [8,9]. For instance, prion-infected neuronal cells released exosomes that significantly increased levels of miR-128a, miR-21, miR-222, miR-29b, miR-342-3p and miR-424 compared with non-infected cell exosomes [10]. The miR-29a, miR-150, miR-518 and miR-875 were up-regulated by a range of 16- to 44-fold in the HIV-infected cell exosomes [11]. These differentially expressed miRNAs were shown to be involved in virus entry, replication and propagation [12,13]. Up to now, the profiling of exosomal miRNAs in SPPV infection is still unknown.

The study herein identified the exosomes released by SPPV-infected

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ovine testicular cells and comparatively analyzed differential expression of loaded miRNAs, which may allow us further to investigate their roles in SPPV pathogenesis.

2. Materials and methods

2.1. Ovine testicular cell isolation and SPPV inoculation

Testes were collected from 2 to 3 month-aged lamb from a local abattoir, Xijiang, and washed five to ten times in phosphate-buffered saline (PBS) containing 100 U/mL penicillin and 100 mg/mL streptomycin. The tunica albuginea was peeled with sterile forceps in a biosafety cabinet, and the testicular tissues were cut into 1 - 2 mm^3 pieces and washed twice in PBS. Then the small tissues were digested in 5–6 fold tissue volumes of 0.25% trypsin at 37 °C for 25 min–30 min, followed by addition of 2 mL fetal bovine serum (FBS) to stop digestion. The digested cells were filtered by 200 μm mesh sieve and centrifuged by 1500 r/min for 5 min as previously reported [14]. The cells were resuspended with DMEM median containing 10% FBS and 4 mM-glutamine, and then cultured at 1.0×10^5 cells/mL in 35 mm dishes for 8 h at 37 °C, 5% CO_2, to allow cells to adhere. After suspended cells were discarded, the remaining adherent cells were washed with PBS three times and DMEM containing 10% FBS was added to continue cultivation.

The SPPV strain XJSW-001 used was isolated from 4-month-aged sheep at Shawan County, Xinjiang Autonomous Region, in 2015, which was passaged five generations in ovine testicular cells and stored in liquid nitrogen in our lab. Prior to virus inoculation, monolayer ovine testicular cells were cultured until the confluence reached 70%–80%, washed three times in PBS, infected by SPPV with one multiplicity of infection (MOI) and then incubated at 37 °C for 2 h. The supernatant was removed, and 7 mL of fresh medium without FBS were added and further cultured for 4 h. Uninfected cells were used as a control. Each group was performed in quadruplicates.

2.2. Exosome isolation

Exosomes were isolated and purified from SPPV infected ovine testicular cell culture supernatant 0 h, 24 h and 72 h post infection according to the following protocol. Briefly, the culture supernatant was centrifuged at 3200 × g for 5 min to remove cells and cellular debris. The supernatant was filtered with a 0.22 μm filter. Then, the filtrate was added 0.5 volumes of Total Exosome Isolation Reagent (Invitrogen) and mixed thoroughly. The mixture was incubated overnight at 4 °C, followed by centrifugation at 10,000 × g for 1 h at 4 °C. The supernatant was removed and the final pellet containing exosomes was resuspended in 500 μl filtered PBS.

2.3. Exosome identification

Exosome samples were diluted in filtered PBS to measure their size distribution using ZETASIZER NANO (Malvern, UK) and nanoparticle tracking analysis software (Version 5.0). Exosome morphology was observed by transmission electron microscopy (TEM). For TEM, 20 μl of exosome suspension were added onto a copper-coated grid for 5 min, stained with 3% phosphotungstic acid solution for 5 min and then dried at 65 °C at 15 min. Exosomes were observed under Hitachi H-600 TEM.

2.4. Total RNA isolation from exosomes

Exosomal RNA was extracted using TRIzol LS Reagent according to the manufacturer's protocol. Briefly, 0.75 mL of TRIzol LS Reagent was added into 0.25 mL of exosome samples resuspended in filtered PBS and incubated at room temperature for 3–5 min, followed by addition of 0.2 mL of chloroform. After the mixture was centrifuged at 12,000 × *g* for 15 min at 4 °C, the aqueous phase supernatant was transferred to a new RNase-free EP tube, and then 10 μg RNase-free glycogen and 500 μL of isopropanol were added, followed by incubation overnight at -20 °C. The mixture was centrifuged at 12,000 × *g* for 10 min at 4 °C, and the RNA-containing pellet was washed with 1 mL 75% ethanol. After centrifuged at 7500 × *g* for 10 min at 4 °C, the pellet was air-dried for 5 min and then dissolved in 40 μL of RNase-free water. The concentration of the isolated RNA was measured by Nanodrop (Invitrogen).

2.5. RNA deep-sequencing and data analysis

RNA samples were sent to BGI (Beijing Genomics Institute) for deep-sequencing. Small RNA libraries were conducted as follows. Briefly, the small RNA fragments in a size of 18 bp to 30 bp were isolated and purified using a PAGE gel. Purified RNAs were ligated with 3′ and 5′ adaptors, and then RT-PCR enrichment was performed by ABI StepOnePlus Real-Time PCR System. The PCR products were purified using 10% PAGE and used for deep-sequencing.

After deep sequencing, the reads with poly A, shorter than 18 nt, and without the insert tags were removed from the raw data. Meanwhile, the reads derived from tRNAs, rRNAs, snoRNAs and snRNAs, repetitive sequences and small degraded mRNAs were also removed as previously described [15]. Afterwards, the clean reads were obtained and used for further analysis. First, these clear sequences were mapped to sheep genome (Version: oviAri3) using SOAP v1.11 software to analyze their location and distribution on the genome [16]. Then the mapped reads were used for identification of known miRNAs by blast-searching against miRNA database (Release 21), and miRDeep was used to identify novel miRNAs with default options [16]. Finally, the target prediction for all differential known miRNAs was conducted using TargetScan, RNAhybrid and miRanda. Gene ontology (GO) and KEGG analyses for identified target genes were also performed.

2.6. qPCR analysis

Exosomal RNA was extracted using TRIzol reagent (Invitrogen) as described above with the following modifications. After mix of 0.75 mL TRIzol LS Reagent and 0.25 mL exosome, 8 μL of *Caenorhabditis elegans* miR-39 (approximately 267 fmol, QIAGEN) as an exogenous reference were added. Prior to RNA precipitation, 1 μL glycogen (10 mg/mL, Invitrogen) was added into RNA-containing aqueous supernatant.

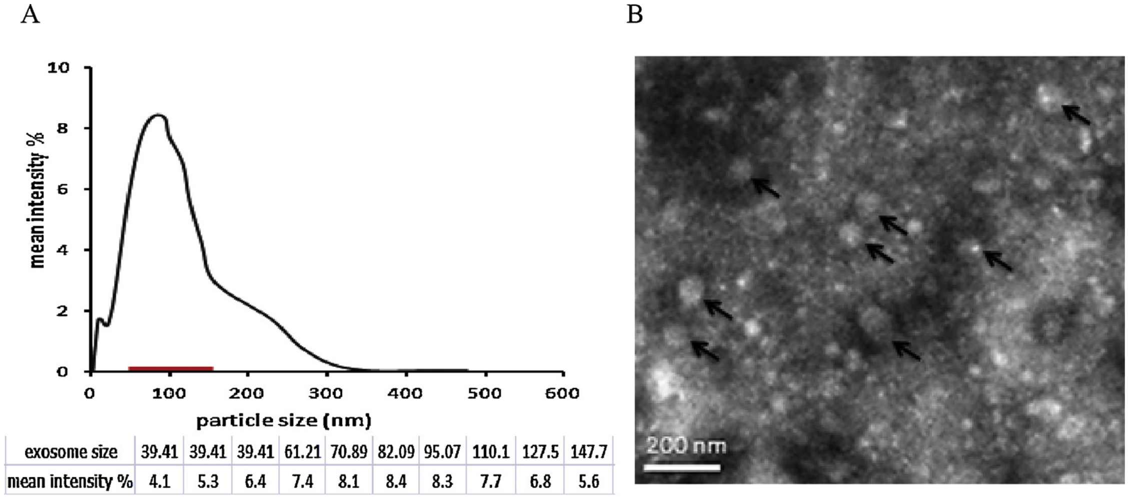
Using the RT primer (Table 1),1 μg of exosomal RNA was used for first stranded cDNA synthesis with All-in-One miRNA First Strand cDNA Synthesis Kit (GeneCopoeia) according to the instructions. After reaction, cDNA products were diluted by addition of 5 volumes of nuclease-free water, and immediately used for qPCR analysis. All-in-One qPCR mix (GeneCopoeia) was used to set up the reactions with the universal primer and the primer specific for each miRNA (Table 1), and qPCR was performed using an ABI7500 thermocycler (ThermoFisher Scientific) with the specific primers (Table 1) according to the two-step protocol: denaturation at 95 °C for 10 min, 40 cycles of 95 °C for 15 S and 60 °C for 1 min. To verify the high-throughput sequencing, eight miRNAs

Table 1

The primers used in this study.

Primer	Sequence (5'-3')
RT primer	GCGAGCACAGAATTAATACGACTCACTATAGG$(T)_{12}$VN[a]
universal primer	GCGAGCACAGAATTAATACGAC
oar-let-7f	TGAGGTAGTAGATTGTATAGT
oar-let-7b	TGAGGTAGTAGGTTGTGTGGT
oar-miR-221	AGCTACATTGTCTGCTGGGTTT
oar-miR-21	TAGCTTATCAGACTGATGTTGAC
oar-miR-10b	ACCCTGTAGAACCGAATTTGTG
oar-miR-27a	TTCACAGTGGCTAAGTTCCG
oar-miR-23a	ATCACATTGCCAGGGATTTCC
oar-miR-26a	TTCAAGTAATCCAGGATAGGCT

[a] 'V' stands for A, G or C; 'N' stands for A, T, G or C.



exosome size	39.41	39.41	39.41	61.21	70.89	82.09	95.07	110.1	127.5	147.7
mean intensity %	4.1	5.3	6.4	7.4	8.1	8.4	8.3	7.7	6.8	5.6

Fig. 1. Exosome morphology.
Exosomes were isolated and purified from SPPV-uninfected and -infected ovine testicular cell culture supernatant 0, 24 and 72 h post infection. Exosome size and morphology were determined by ZETASIZER (A) and TEM (B, arrowed), respectively.

including oar-let-7f, oar-let-7b, oar-miR-27a, oar-miR-23a, oar-miR-221, oar-miR-21, oar-miR-10b and oar-miR-26a were selected for validation. The relative miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ formula method and normalized to that of cel-miR-39.

2.7. Statistical analysis

Statistical analysis was conducted using GraphPad Prism 5 and a One-way ANOVA was used for comparison of miRNA expression levels 0 h, 24 h and 72 h post infection. If *p* value was less than 0.05, the difference was considered to be significant.

3. Results

3.1. Exosome morphology

The size of all the isolated exosomes mainly ranged from 40 nm to 130 nm in diameter (Fig.1A). TEM observation also revealed a population of spherical particles in a diameter of 30 nm–150 nm in all samples (Fig. 1B).

3.2. Brief data presentation of three small RNA sequencing libraries

A total number of 28,886,828, 28,286,194 and 29,491,460 reads were generated from the control and two SPPV-infected samples, respectively. After low-quality reads were removed, more than 85% clean reads from three samples were retained and used for downstream analysis, and more than 61% of reads were mapped to the *O. aries* genome (Table 2).

3.3. Identification of differentially expressed exosomal miRNAs

A total of 385 miRNAs ranged from 18 bp to 24 bp were detected in three exosomal samples (Table S1). Of them, 106 were known miRNAs and 279 were novel miRNAs. 78 known and 54 novel miRNAs were commonly detected in exosomes released by both SPPV-infected ovine testicular cells (24 h and 72 h) and uninfected cells (Fig. 2A and B). Compared with the exosomes by the uninfected controls, a total of 34 miRNAs were aberrantly expressed in the exosomes from infected cells (Fig. 2C). For example, oar-let-7b, oar-miR-27a, oar-miR-23a, oar-miR-221 and oar-miR-16b were significantly down-regulated, and oar-miR-21, oar-miR-10b, oar-miR-26a and oar-let-7 g were significantly up-regulated.

Of 8 miRNAs validated, 5 including oar-miR-21, oar-miR-10b, oar-let-7f, oar-let-7b and oar-miR-221 were confirmed to be consistent with the sequencing data in expression. The expression of oar-miR-21 and oar-miR-10b was shown to be the highest in exosomes at 24 h after SPPV-infected, and the expression of oar-let-7f was the highest in exosomes at 72 h. Conversely, the expression of oar-let-7b and oar-miR-221 was significantly decreased 24 h and 72 h post infection compared with SPPV-uninfected (Fig. 3). In disagreement with the sequencing data, oar-miR-27a and oar-miR-23a were upregulated 24 h post infection, while oar-miR-26a expression kept constant in all three exosomal samples (data not shown).

3.4. Target genes of differentially expressed known miRNAs

A total of 953,586 potential targets for these dysregulated known exosomal miRNAs were obtained, 377,674 of which were commonly predicted by three different software. The GO enrichment analysis revealed that more than 51.1% of predicted genes were involved in biological processes, approximately 37.5% being involved in cellular component and 11.4% in molecular function (Fig. 4A). It was also found that these differentially expressed miRNAs were involved in immune system processes and stimulus responses (Fig. 4A). The predicted target genes were mostly associated with MAPK signaling

Table 2
Data summary of three small RNA sequencing libraries.

	Uninfected	Infected 24h	Infected 72h
Total reads	28,886,828	28,286,194	29,491,460
Clean reads	24,564,754 (85.0%)	25,064,095 (88.6%)	26,685,806 (90.5%)
Reads mapped to the genome	19,129,073 (77.9%)	18,555,309 (74.0%)	16,361,427 (61.9%)
Number of miRNAs identified	296	271	201
Total reads of miRNAs	3,235,367	2,589,046	913,245

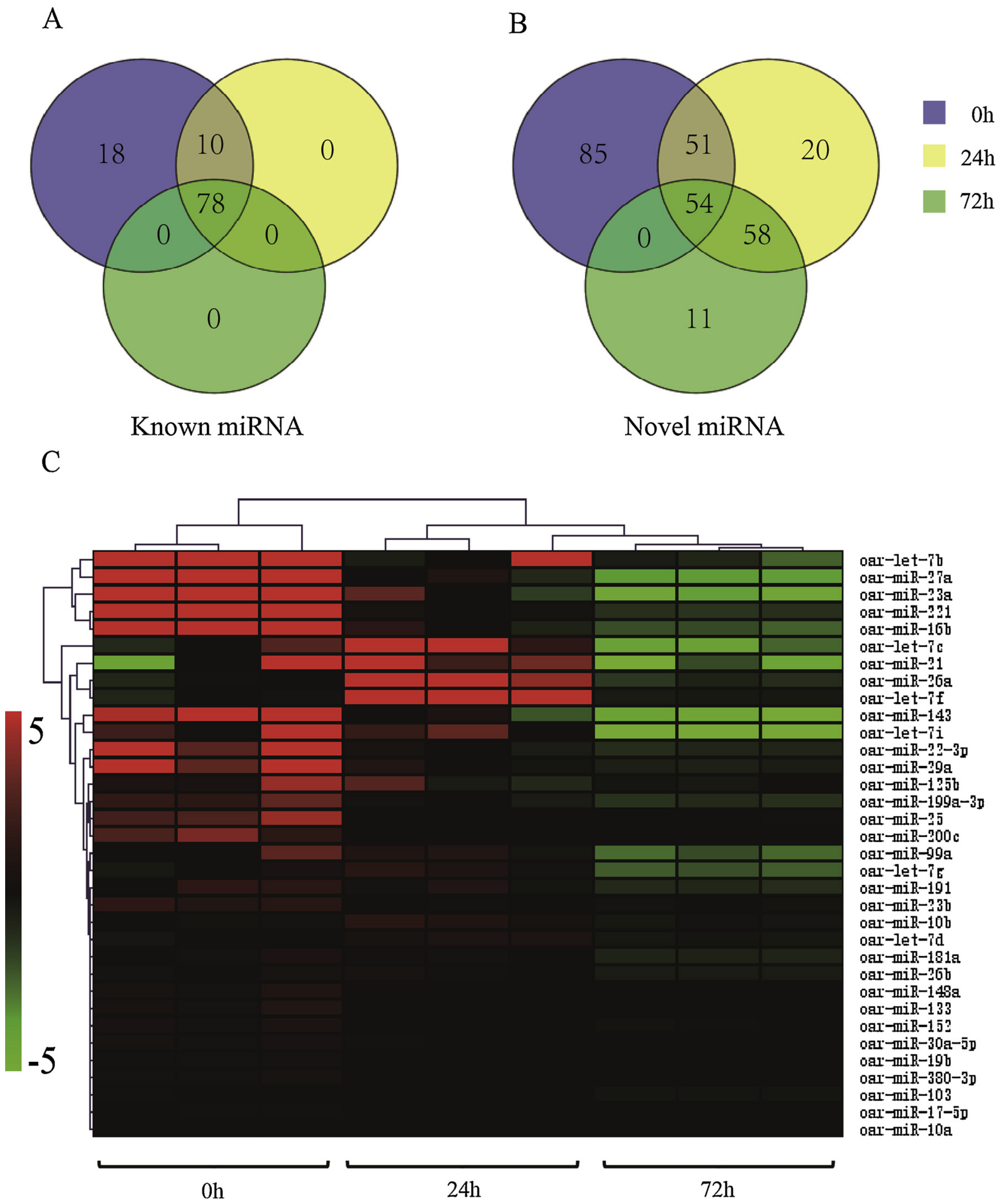


Fig. 2. Comparative analysis of differentially expressed exosomal miRNAs.
(A) Known miRNAs identified in the infected and uninfected groups.
(B) Novel miRNAs identified in the infected and uninfected groups.
(C) Clustering of differentially expressed known exosomal miRNAs.

pathway, autophagy, Hipoo signaling pathway and so on (Fig. 4B).

4. Discussion

SPPV, which is a kind of double-stranded DNA virus and replicates in the cytoplasm of host cells, contains approximately 147 putative genes [17]. SPPV infection can lead to a relative decrease of the vast majority of host RNA in expression. For vaccinia virus, a type of poxvirus, the synthesis of viral DNA reached at the peak 24 h post infection [18]. This rapid change can cause the profound shutdown of host protein synthesis and repression of antiviral responses. Therefore, we harvested and investigated exosomes at 24 h and 72 h after infection. It is well known that ovine testicular cells are optimal host cells for SPPV *in vitro* culture, due to the high sensitivity [2]. Therefore, ovine testicular cells were selected in this study to explore the miRNA profiles of exosomes in the process of SPPV infection.

Accumulating evidence has demonstrated that host exosome pathways are hijacked by viruses and the virally modified exosomes contribute to virus spread and immune evasion [19]. Meanwhile, exosomes can escape host recognition to prevent from stimulation of the immune

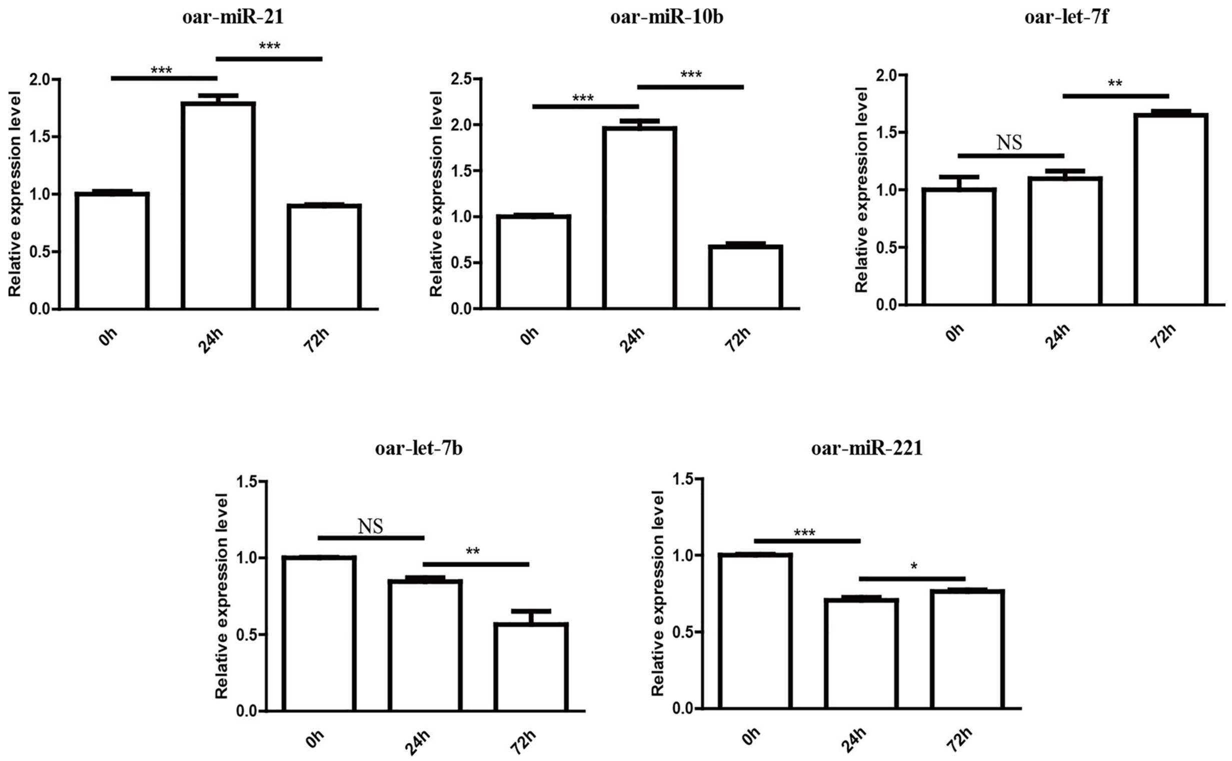


Fig. 3. Validation of miRNA expression by qPCR assay.

An One-way ANOVA test was used for comparison of miRNA expression levels 0 h, 24 h and 72 h post infection and data were expressed as means ± SEM. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$; NS: not significant.

responses and to protect their internal active substances to be degraded in the blood and tissue fluids in a long distance transportation process [20]. It has been shown that exosomes can protect virions from the action of neutralizing antibodies, thus increasing their stability in the spread [21]. The virally modified exosomes have effects on host cells in a number of ways. For example, inhibition of endogenous HPV18 E6/E7 expression led to the alterations of miRNA composition in exosomes secreted by cervical cancer cells and these E6/E7-dependent exosomal

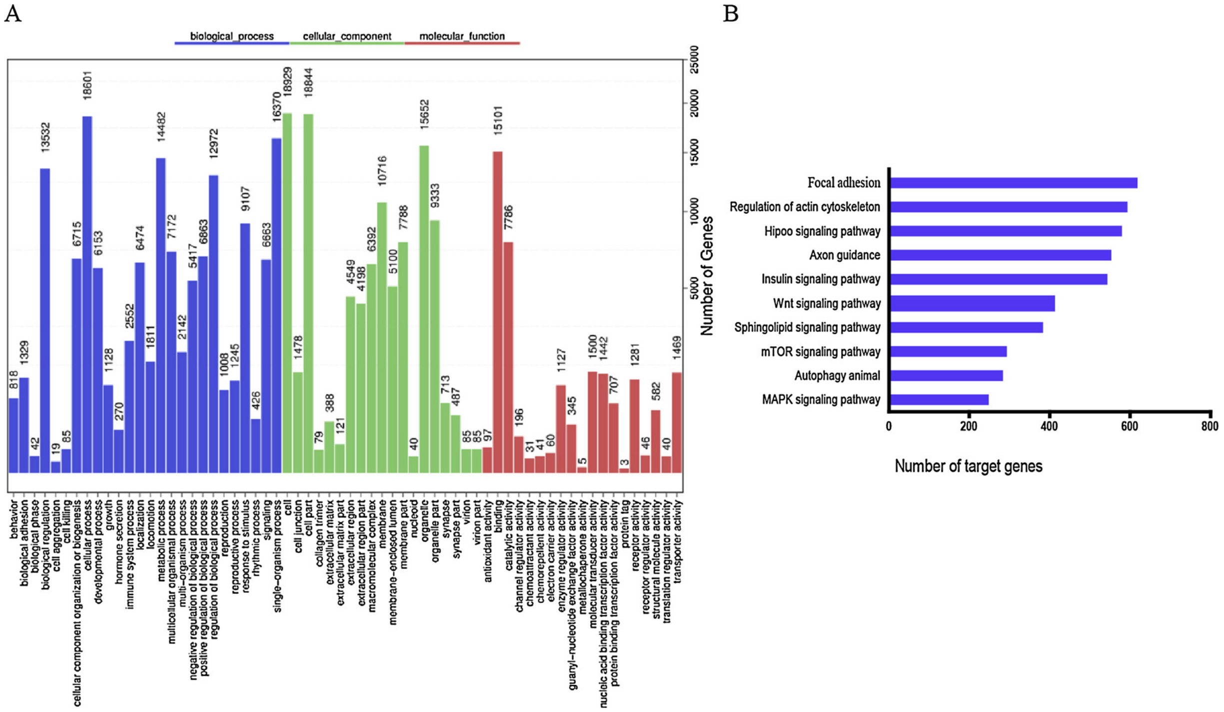


Fig. 4. GO and KEGG pathway analysis.

(A) GO analysis of target genes of the dysregulated miRNAs.

(B) Top 10 KEGG pathways of the target genes of differentially expressed miRNAs.

miRNAs were further found to be involved in regulation of cell proliferation and apoptosis [22]. For KSHV, infected cells were demonstrated to specifically transfer the virus encoded miRNAs to surrounding cells via exosomes, and this exosome-mediated metabolic reprogramming of neighboring cells supports the growth of infected cells, thereby contributing to viral adaptive abilities [23].

In this study, we found that SPPV infection perturbed exosomal miRNA expression, and identified many miRNAs being significantly differentially expressed. Previous reports have demonstrated that mammalian poxviruses [24] and vaccinia virus [25] encode poly(A) polymerase, which polyadenylates viral transcripts and mediates 3′ polyadenylation of host miRNAs, leading to host miRNA degradation and promote poxvirus replication by host machinery. In other words, miRNA precursors in the infected cells may be degraded by poxvirus-induced polyadenylation. In our analysis, almost all miRNAs were down-regulated at 72 h after infection. Therefore, it is interesting to explore the effect of this polyadenylation on exosomal miRNAs during SPPV infection. At the same time, qPCR analysis showed that SPPV infection induced exosomal oar-miR-21 upregulation. The current study has demonstrated that EV71 evades the immune surveillance system to proliferate by activating miR-21, thus promoting EV71 replication [26]. Another study also indicated that miR-21 promotes dengue virus serotype 2 replication in HepG2 cells [27]. Whether exosomal oar-mir-21 can promote SPPV replication will be of interest to be further explored in future experiments.

In general, one miRNA has hundreds to thousands of predicted target genes and one target gene can be modulated by multiple miRNAs [28]. In order to further understand the potential roles of differentially-expressed exosomal miRNAs, all predicted mRNA transcripts were classified and annotated using GO database. The predicted target genes are mostly involved in mTOR signaling pathway, indicating its critical functions in defensing SPPV infection. It has been found that both host antiviral responses and poxvirus countermeasures rely upon the control of the mTOR pathway, and poxviruses evade cytosolic sensing through disruption of an mTORC1-mTORC2 regulatory circuit [29]. Of interest is to investigation the association between these differentially expressed exosomal miRNAs and the mTOR signaling pathway in future studies.

In summary, we have identified a set of dysregulated miRNAs in exosomes released by SPPV-infected ovine testicular cells. In future studies, the functions of these dysregulated miRNAs are required to be explored, which may help us to clarify the mechanisms of SPPV-host interactions.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2019.101363.

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