Release of extracellular vesicles containing small RNAs from the eggs of *Schistosoma japonicum*

Shanli Zhu¹, Sai Wang¹, Yu Lin¹, Pengyue Jiang¹, Xiaobin Cui¹, Xinye Wang¹, Yuanbin Zhang¹ and Weiqing Pan¹,²*

Abstract

**Background:** *Schistosoma japonicum* is a parasitic flatworm that causes human schistosomiasis. Secreted extracellular vesicles (EVs) play a key role in pathogen-host interfaces. Previous studies have shown that *S. japonicum* adult worms can release microRNA (miRNA)-containing EVs, which can transfer their cargo to mammalian cells and regulate gene expression in recipient cells. Tissue-trapped eggs are generally considered the major contributor to the severe pathology of schistosomiasis; however, the interactions between the host and parasite in this critical stage remain largely unknown.

**Methods:** The culture medium for *S. japonicum* eggs in vitro was used to isolate EVs. Transmission electron microscopy (TEM) analysis was used to confirm that vesicles produced by the eggs were EVs based on size and morphology. Total RNA extracted from EVs was analyzed by Solexa technology to determine the miRNA profile. The in vitro internalization of the EVs by mammalian cells was analyzed by confocal microscopy. The presence of EVs associated miRNAs in the primary hepatocytes of infected mice was determined by quantitative real-time PCR (qRT-PCR).

**Results:** EVs were isolated from the culture medium of in vitro cultivated *S. japonicum* eggs. TEM analysis confirmed that nanosized vesicles were present in the culture medium. RNA-seq analysis showed that the egg-derived EVs contained small non-coding RNA (snRNA) populations including miRNAs, suggesting a potential role in host manipulation. This study further showed that Hepa1-6, a murine liver cell line, internalized the purified EVs and their cargo miRNAs that were detectable in the primary hepatocytes of mice infected with *S. japonicum*.

**Conclusions:** *Schistosoma japonicum* eggs can release miRNA-containing EVs, and the EVs can transfer their cargo to recipient cells in vitro. These results demonstrate the regulatory potential of *S. japonicum* egg EVs at the parasite-host interface.

**Keywords:** *Schistosoma japonicum*, Eggs, Extracellular vesicles, Small non-coding RNAs, miRNAs

Background

Schistosomiasis is a serious parasitic disease throughout the world’s tropical regions, affecting more than 230 million people according to conservative estimates [1]. Three main species of schistosomes infect humans, including *Schistosoma haematobium*, *Schistosoma mansoni* and *Schistosoma japonicum*. In China, *S. japonicum* remains a major public health problem, although considerable efforts have been devoted to controlling its transmission over the last few decades [2–4]. The complex life-cycle of schistosomes involves multiple developmental stages, including the egg, miracidium, cercaria, schistosomulum and adult worm. Tissue-trapped eggs are generally considered the major contributor to the severe pathology of schistosomiasis, because the parasite has developed strategies to both facilitate the establishment of infection and evade or manipulate the host immune response. Recent investigations have made some progress in the analysis of parasite-host interactions [5–8]. However, a deeper understanding of mechanisms of parasitism is urgently required for discovering novel interventions to control the disease.
Exosomes are a subtype of small (30–150 nm in diameter), membrane-enclosed vesicles, released by various types of mammalian cells in both normal and pathological conditions [9–11]. These vesicles originate from the inward budding of endosomal membranes, which creates multivesicular bodies that fuse with the plasma membrane to effect release [12, 13]. Originally assumed to be cellular waste products, exosomes are now considered highly bioactive extracellular vesicles (EVs) that facilitate intracellular communication [14]. The cargo of exosomes is complex and variable, containing bioactive proteins, functional miRNAs, miRNAs and other small non-coding RNA (sncRNA) species [9, 10, 15]. Therefore, these vesicles can “horizontally” transfer signals to neighboring cells and thus serve as mediators of intercellular communication [15–20]. The discovery of EVs in plants, fungi and bacteria drove the theory that exosome-mediated communication could be operated on a cross-species basis [21–23]. Recent studies of Heligmosomoides polygyrus, Echinostrongylus caproni, Fasciola hepatica, Dicrocoelium dendriticum, Brugia malayi and Leishmania donovani have demonstrated that EVs are secreted from parasites and may be taken up by host cells [24–29]. The vesicles produced by parasites can deliver virulence factors (proteins and non-coding RNAs) to the host, thus favoring pathogen survival and disease progression [24, 27, 29]. In addition, characterization of EVs released by S. mansoni schistosomula [30], S. mansoni adult worms [31] and S. japonicum adult worms [32, 33] suggested that EVs and their cargo could play a key role in parasite-host interplay. Based upon the increasing realization that EVs facilitate intercellular communication in eukaryotes, we speculate that they contribute to maintenance of the long-term host-parasite interactions during schistosomiasis.

Here, we show that the egg of S. japonicum releases EVs whose size and morphology are consistent with exosomes. These vesicles contain small RNAs, including parasite-specific miRNAs and conserved miRNAs that contain identical seed sites between the host and S. japonicum. Further studies showed that the secreted EVs are internalized by mammalian cells and their cargo miRNAs can be transferred to the recipient cells. Moreover, the egg EVs associated-miRNAs can be detected in the primary hepatocytes of mice infected with S. japonicum. These results suggest the regulatory potential of S. japonicum egg EVs at the parasite-host interface.

**Methods**

**Schistosoma japonicum egg isolation, culture and culture medium collection**

For collection of S. japonicum egg secretion products, New Zealand rabbits were percutaneously infected with approximately 1,200 S. japonicum cercariae that were shed from lab-infected snails (Oncomelania hupensis) obtained from the National Institute of Parasitic Disease, Chinese Center for Disease Control and Prevention. Eggs were isolated in sterile conditions from the livers of infected rabbits 6–7 weeks post-infection using the sieving and enzymatic method described by Cai et al. [34]. After collection, the freshly isolated eggs were thoroughly washed three times with PBS (pH 7.4) and then maintained in preheated RPMI-1640 culture medium (Life Technologies, Carlsbad, CA, USA) containing 100 U of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml amphotericin B (Life Technologies) at 37 °C under 5 % CO₂ at a density of 1 x 10⁴ eggs/ml for 24 h. Following 24 h incubation, eggs and pellets were removed by centrifugation at 3,000 x g at 4 °C. The culture medium was collected for further exosome isolation as described below.

**Exosome-like vesicle isolation**

An ExoQuick-TC Exosome Precipitation Kit from System Bioscience (SBI, Mountain View, CA, USA) was used for exosome isolation according to the supplier’s protocols. In brief, the medium treated as described above was initially centrifuged at 3,000 x g for 15 min to remove remaining eggs and cell debris. Exosome isolation reagents (0.2 volumes) were added to the supernatants and incubated at 4 °C overnight. The ExoQuick-TC/medium mixture was then centrifuged at 15,000 x g for 30 min at room temperature. The supernatants were discarded and pellets were resuspended in 50 μl PBS and stored at -80 °C until further analysis.

**Transmission electron microscopy**

For visualization of the vesicles, purified EVs were applied to 200 mesh formvar-coated EM grids (Agar Scientific, Essex, UK), incubated for 1 min at room temperature, and then excess sample was removed. The grids were stained with 1 % uranyl acetate (SBI) for 5 min. The grids were then loaded onto the sample holder of the JEM-1230 TEM (JEOL, Tokyo, Japan) and exposed to an 80 kV electron beam for image capture.

**Small RNA library preparation and analysis**

For detection of RNA species, total RNA was extracted from EVs released by parasite eggs using Trizol (Life Technologies), and RNA quality was evaluated with an Agilent Technologies 2100 system (Agilent Technologies, Santa Clara, CA, USA). The small RNAs between 18 and 30 nt were isolated by 15 % polyacrylamide gel electrophoresis (PAGE) and then ligated into Illumina’s proprietary 5’ and 3’ adaptors, and further converted into single strand cDNA. The cDNA products were amplified using a 3’-adaptor reverse primer and 5’-adaptor forward primer (Illumina, San Diego, CA, USA). The purified PCR products were used for clustering and sequencing by an Illumina Genome Analyzer at the Beijing Genomics Institute, Shenzhen.
All unique sequences along with their associated read counts were determined from the raw data. First, low-quality reads, 3′-adapter null reads, insert null reads, 5′-adapter contaminants and reads with polyA tails were filtered. The clean datasets were mapped to the draft S. japonicum genome sequences (sjr2_scaffold.fasta, downloaded from ftp://lifecenter.sgst.cn:2121/nucleotide/corenucleotide) using the Short Oligonucleotide Alignment Program (http://soap.genomics.org.cn). We investigated the length distribution of the perfectly matched small RNA reads in the libraries. By comparing our sequences with the non-coding RNAs collected in Rfam (Version 11.0) and the NCBI GenBank database, sequenced small RNAs were annotated to different categories, including tRNAs, small nuclear RNAs (snRNAs), repeat associated small RNAs, tRNAs and miRNAs. For miRNA analysis, the unmatched small RNAs were further analyzed against miRBase (version 21) and GenBank to identify known mature miRNAs. Finally, unannotated small RNAs were used for novel miRNA prediction analysis using Mireap (http://sourceforge.net/projects/mireap). RNAfold was used to predict hairpin-like structures.

**Quantitative real-time PCR (qRT-PCR) analysis of S. japonicum egg EV associated miRNAs**

Stem-loop qRT-PCR was used to validate the presence of miRNAs in S. japonicum egg EVs. Total RNA of egg EVs, culture medium and residuum supernatants (collected after EV isolation) were extracted using Trizol. qRT-PCR analysis was performed as previously described [35]. Briefly, a stem-loop RT primer was used to reverse-transcribe mature miRNA to cDNA; the 10 μl reverse transcription reactions contained 150 ng of total RNA, 50 nM of each individual stem-loop RT primer, 0.5 mM dNTP (Takara), and 5 U M-MLV reverse transcriptase (Takara), and 2 U RNase inhibitor. The temperature program was 60 min at 42 °C, 15 min at 70 °C and then holding at 4 °C. qRT-PCR was performed using an Applied Biosystems (ABI, Carlsbad, CA, USA) 7500 Sequence Detection system. The 20 μl PCR reaction included 2 μl of RT product (1:1 dilution), 1 × SYBR Premix Ex Taq (Takara), 0.5 μM specific forward primer, and 0.5 μM common reverse primer. The reactions were performed at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Cel-miR-39 was used as an internal control, and the relative miRNAs level was calculated by the $2^{-ΔΔCt}$ method [36]. All reactions were run in triplicate. All primers used are listed in Table 1.

### Confocal analysis of S. japonicum egg EV uptake by murine liver cells

Murine liver Hepa1-6 cells were obtained from the ATCC (CRL-1830) and grown according to the standard protocol in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies) supplemented with 10 % fetal bovine serum (Life Technologies). Hepa1-6 cells were seeded in 12-well plates (1 × 10^5 cells/well) using advanced serum-free DMEM (Life Technologies) for 4 h. Purified EVs from S. japonicum eggs or Hepa1-6 cells were labeled with the green fluorescent dye PKH67 (Sigma-Aldrich, St. Louis, MO, USA) as described by Hazan-Halevy et al. [37] with minor modifications. Briefly, 10 μg of the PKH67-stained EVs were washed three times using a 300-kDa Amicon (Merck Millipore, Merck KGaA, Darmstadt, Germany) to remove excess dye, EVs were then added to the cells and incubated for 1 h at 37 °C. As a control for non-specific labeling of cells, PBS was stained with PKH67, washed, and added to the cells. Following 1 h incubation, the medium was aspirated, cells were washed twice with PBS, fixed with 4 % formaldehyde solution for 15 min, and washed twice more with PBS; nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies). Finally, the cells were observed using confocal fluorescence microscopy (Leica TCS SP5 II, Heidelberg, Germany).

### qRT-PCR analysis of Schistosoma-specific miRNAs

Hepa1-6 cells were incubated with EVs (10 μg total protein per well) derived from S. japonicum eggs or Hepa1-6 cell culture medium (negative control) for 20 h. After washing twice with PBS, the cells were then incubated with 0.25 % trypsin/EDTA (Life Technologies) for 3 min to eliminate non-specifically adhered EVs. Total RNA was extracted from the incubated cells using Trizol, and miRNAs were reverse transcribed into cDNA using Reverse Transcriptase M-MLV (Takara) as described above. Real-time PCR was performed to assess the Schistosoma-specific miRNAs in the treated Hepa1-6.

| Table 1 Sequences of primers used for qRT-PCR |
| --- |
| Gene | Name | Sequence (5′-3′) |
| sja-miR-71b | RT stem-loop primer | CTCAACTGGTGTCGTGGAGTCGG |
|  | Forward primer | AAATATTAGGCAAGGTCGTCA |
| sja-bantam | RT stem-loop primer | CTCAACTGGTGTCGTGGAGTCGG |
|  | Forward primer | AAATATTAGGCAAGGTCGTCA |
| cel-miR-39 | RT stem-loop primer | CTCAACTGGTGTCGTGGAGTCGG |
|  | Forward primer | AAATATTAGGCAAGGTCGTCA |
| mmu-U6 | Forward primer | CTTCGGCGACACATATACTAA |
|  | Reverse primer | CGGTCCAGATTTGCAGGTTCAT |
cells. Cel-miR-39 was used as an internal control, and the relative miRNAs level was calculated by the $2^{-\Delta C_t}$ method.

**Isolation of primary mouse hepatocytes**

To ascertain whether parasite-specific miRNAs were present in the primary hepatocytes of mice infected with *S. japonicum*, primary mouse hepatocytes were isolated by a two-step collagenase perfusion procedure, as described by He et al. [38] with minor modifications. Briefly, 6-week-old male C57BL/6 mice were purchased from the experimental animal center of the Second Military Medicine University and exposed percutaneously to 20 *S. japonicum* cercariae. Livers of *S. japonicum*-infected mice (49 days post-infection (dpi) and 80 dpi, n = 5, respectively) and uninfected mice were initially in situ digested with 0.04 % collagenase type IV and then further digested with 0.08 % collagenase type IV at 37 °C in a shaking bath for 30 min. The single cell suspensions were harvested by filtration through 400-mesh sieves for removal of the remaining tissue debris and parasite eggs. Next, hepatocytes were isolated by centrifugation of the resulting cell suspensions at 50× g for 4 min and further purified by centrifugation at 20× g for 4 min. Purified hepatocytes were resuspended in DMEM containing 20 μg/ml Ribonuclease A (Sigma-Aldrich) at 37 °C in a bath for 30 min to eliminate the miRNA released by schistosome eggs. After three times washing with PBS, the cell pellet was used immediately for extraction of total RNA or stored in liquid nitrogen until further analysis.

**qRT-PCR analysis of *S. japonicum* miRNAs in primary hepatocytes of infected mice**

Total RNA of primary hepatocytes derived from infected and uninfected mice were extracted using Trizol, and parasite miRNAs were reverse transcribed into cDNA using reverse transcriptase M-MLV as described above. qRT-PCR analysis was performed to determine the presence of parasite-specific miRNAs in the primary hepatocytes of infected mice. The U6 gene was used as an internal control. The $2^{-\Delta C_t}$ method was used to calculate the level of miRNAs.

**Statistical analysis**

Results were analyzed using SPSS software (version 16) and statistical analyses were performed using one-way ANOVA. A value of $P < 0.05$ was considered statistically significant. Data are expressed as mean ± standard deviation.

**Results**

**Isolation and purification of schistosomal eggs**

Rapid isolation of viable schistosomal eggs from host hepatic tissue in sterile conditions was a critical step for obtaining EVs. In this study, eggs were isolated and purified from infected rabbits using the sieving and enzymatic methodology described by Cai et al. [34]. The purified egg samples were examined under a light microscope, and we observed that most of the eggs contained a developing embryonic larva (Additional file 1: Figure S1).

**EVs isolation from schistosomal eggs and small RNA analysis**

The schistosomal eggs were incubated for 24 h in standard culture conditions, and vesicles were purified from the culture medium and evaluated by TEM. TEM images showed that eggs cultivated in vitro secreted abundant 30–100 nm microvesicles consistent with the morphology previously described for *S. japonicum* adult worms [33], confirming that schistosomal eggs also release EVs (Fig. 1).

ScnRNAs carried by EVs function as messengers for intercellular communication. Therefore, we identified the small RNA populations associated with schistosomal egg EVs by Solexa deep sequencing. As shown in Fig. 2a, b, the length distribution of schistosomal egg EV-associated small RNAs ranged from 18 to 30 nt. rRNAs, snRNAs and repeat associated small RNAs were the dominant classes of small RNAs, followed by intron sense, tRNAs and miRNAs (Fig. 2c). We found 13 known *S. japonicum* miRNAs (reads >100) present in the schistosomal egg EV libraries (Table 2 and Additional file 2: Table S1), including three miRNAs (miR-10, bantam and miR-3479-3p) that were present in the plasma of *S. japonicum* infected host rabbits in a previous study [39]. In addition, one novel miRNA was predicted using Mireap (Table 2 and Additional file 2: Table S1). Then, stem-loop qRT-PCR was performed to verify the presence of *Sja*-bantam and *Sja*-miR-71b in the RNA isolated from schistosomal egg EVs (Fig. 2d). These findings demonstrated that schistosomal eggs release EVs during development in vitro and these 30–100 nm sized vesicles carry miRNAs that are both parasite-specific and homologs of mammalian (host) (e.g. mouse miR-10) miRNAs.

**Uptake of schistosomal egg EVs by murine cells**

To ascertain whether the schistosomal egg-derived EVs can enter mammalian cells, internalization was examined in mouse liver cells. EVs originating from schistosomal eggs or Hepa1-6 cells (positive control) were labeled with the green fluorescent lipid dye PHK67 and incubated with Hepa1-6 cells in vitro. Confocal image analysis revealed efficient internalization of the parasite egg-EVs by murine Hepa1-6 cells (Fig. 3a). qRT-PCR analysis of the treated cells demonstrated that schistosomal egg EVs associated miRNAs (bantam and miR-71b) were detectable in Hepa1-6 cells after 20 h of incubation.
with parasite EVs (Fig. 3b). The above indicated that the miRNAs carried by schistosomal egg EVs can be transferred to recipient mammalian cells.

**qRT-PCR analysis of** *S. japonicum* **miRNAs in the primary hepatocytes of infected mice**

To ascertain whether parasite-specific miRNAs were present in the primary hepatocytes of mice infected with *S. japonicum*, primary hepatocytes of infected mice were isolated and detected for two miRNAs that are associated with *S. japonicum* egg EVs (i.e. Sja-miR-71b and Sja-bantam). The result showed the two miRNAs were detectable in the primary hepatocytes at 49 dpi and 80 dpi (Fig. 4), indicating that parasite-derived miRNAs were present in the primary hepatocytes of infected mice after numerous eggs deposited in the liver.

**Discussion**

Extracellular vesicles (EVs), and particularly exosomes, are emerging as one of the major mediators of intercellular communication within mammalian systems. EVs deliver their cargo molecules to specific cells and the transfer of molecules may modulate cellular activity and pathways in recipient cells. Recent studies revealed that helminth parasites such as *B. malayi*, nematode *H. polygyrus* adults, *S. mansoni* schistosomula and *S. japonicum* adults, can also release EVs enriched in proteins and non-coding RNAs [24, 27, 30, 33, 40]. However, it remained unknown whether *S. japonicum* eggs, the major agents causing the severe pathology of schistosomiasis, could release EVs. Here, we isolated and cultivated the tissue-trapped eggs of *S. japonicum*, and verified that in vitro cultivated eggs released nanosized vesicles into the culture supernatant (Fig. 1). These nanovesicles potentially represent novel modulators of host and parasite interactions, therefore, their snRNA content was explored further.

RNA deep sequencing revealed that miRNAs were among the small RNAs in the schistosomal egg EV libraries. Considering RNAs with > 100 reads, 13 previously characterized miRNAs and one potential novel miRNA were identified. Among the 13 known Sja-miRNAs identified in the egg EVs, Sja-bantam, Sja-miR-10 and Sja-miR-3479-3p were all previously detected in serum obtained from rabbits infected with *S. japonicum* [39]. Also, it has been shown that Sja-miR-277 and Sja-miR-3479-3p were detectable in the serum samples of infected mice [41]. We speculate that the circulating miRNAs are protected from degradation by encapsulation in the EVs. Interestingly, bantam and miR-10 were significantly enriched in the libraries of EVs derived from schistosomal adult worms, whereas miR-3479-3p did not appear in those EV libraries [33]. Moreover, it has been shown that five miRNAs (miR-71, miR-71b, miR-1, miR-36, and miR-124) are the most abundant in the egg stage of *S. japonicum* [34], implying that these miRNAs play important roles in embryo development. In the present study, four of these miRNAs (all except Sja-miR-1) were incorporated into the egg EVs. A similar phenomenon was also observed in the adult stage of *S. japonicum* [33]. These findings support the notion that miRNAs are selectively packaged into exosomes and delivered to specific cells, although it remains unclear whether the sorting mechanism is related to association with the RNA-induced silencing complex components or the target miRNAs [42–44].

In mammals, cell-to-cell communication could be mediated by EVs, which can deliver their cargo miRNAs to recipient cells [13, 14]. Exosomal miRNAs have specific functions such as immune response activation and cell development [45, 46]. The helminthic nematode *H. polygyrus* utilizes exosomal vesicles to increase virulence in a fashion similar to that of the mammalian miRNA transport mechanism [24]. *H. polygyrus* secretes miRNA-loaded vesicles that are accompanied by a nematode AGO protein, most likely to stabilize the miRNAs. Remarkably, *H. polygyrus* vesicles are internalized by mouse cells, which results in suppression of host immunity. Some *H. polygyrus* miRNAs were shown to target in vitro host miRNAs that are related to host immunity. In addition, previous study has shown that
Exogenous plant miRNAs are not only present in serum and tissues of hosts after oral uptake, but they can also regulate the expression of specific genes in the host liver [21]. These findings support the notion of miRNA-mediated cross-kingdom regulation, although the mechanisms responsible for exogenous miRNA transfer and function remain largely unknown. In the present study, we showed that murine liver cells could internalize schistosomal egg EVs and their cargo miRNAs in vitro. The results were consistent with previous findings that EVs derived from nematodes and S. japonicum adults could be taken up by mouse small intestinal epithelial cells and liver cells, respectively [24, 33]. Therefore, EVs derived from S. japonicum eggs potentially act as vehicles to package and deliver miRNAs to host cells and regulate host gene expression, which may facilitate parasitism.

**Fig. 2** Identification of small RNAs associated with *S. japonicum* egg extracellular vesicles (EVs). a Summary of the output of the Solexa data; the percentage in parentheses indicates the percentage of high-quality reads. b The length distribution of small RNA tags. c Classification of the small RNAs by comparison with the *S. japonicum* genome. d qRT-PCR validation of the abundance of Sja-bantam and Sja-miR-71b in the RNA isolated from *S. japonicum* egg EVs. Values are normalized to culture medium, based on equal volumes of starting material. Abbreviations: CM, culture medium; EVs, extracellular vesicles; Residuum, supernatant medium from the final step of EV extraction.

**Table 2** List of identified miRNAs associated with *S. japonicum* egg EVs

| Small RNA ID  | Location | Sequence                                  | Reads<sup>a</sup> | miRNA       |
|--------------|----------|-------------------------------------------|-------------------|-------------|
| t0000052     | SJC_S000027 | 600247–600269                             | 29608             | sja-miR-36-3p |
| t0000207     | SJC_S000052 | 314799–314820                             | 6156              | sja-miR-10-3p |
| t0000255     | SJC_S000254 | 288019–288040                             | 2307              | sja-bantam  |
| t0000729     | SJC_S000054 | 245452–245472                             | 1332              | sja-miR-2a-3p |
| t0000925     | SJC_S000054 | 245576–245597                             | 1085              | sja-miR-71a  |
| t0001363     | SJC_S000055 | 384663–384684                             | 1070              | sja-miR-3479-3p |
| t0001942     | SJC_S000471 | 22294–22314                               | 1038              | sja-miR-2162-3p |
| t0001985     | SJC_S000102 | 364277–364299                             | 746               | sja-miR-71b-3p |
| t0002533     | SJC_S000054 | 245393–245413                             | 585               | sja-miR-2b-3p |
| t0002630     | SJC_S000664 | 24730–24752                               | 570               | sja-miR-61   |
| t0003175     | SJC_S000001 | 925810–925830                             | 554               | sja-miR-277  |
| t0003502     | SJC_S004031 | 7481–7503                                 | 238               | sja-miR-307  |
| t0005635     | SJC_S000102 | 364554–364577                             | 139               | sja-miR-2d-3p |
| t0007134     | SJC_S000110 | 287436–287456                             | 105               | novel-miR-7  |

<sup>a</sup>Only miRNAs with > 100 reads are listed
Several lines of evidence have shown that highly conserved miR-71 and bantam are packaged in parasite-derived EVs, including from *H. polygyrus*, *B. malayi* and *S. mansoni*, suggesting that conserved miR-71- and bantam-secretion systems might exist in helminths [24, 27, 30]. In *Drosophila*, bantam miRNA has been shown to target a tumor-suppress pathway, promoting cellular growth and suppressing cellular apoptosis [47]. Although the role of miR-71 secreted by parasites remains unclear, it has been proposed to be involved in host-pathogen interactions [24, 30]. In the present study, we observed that Sja-miR-71b and Sja-bantam are also incorporated into the EVs derived from schistosomal eggs and these miRNAs can be transferred to murine liver cells in vitro. More importantly, we found that the parasite-specific miR-71b and bantam were present in the primary hepatocytes of *S. japonicum* infected mice after numerous eggs deposited in the liver.

Conclusions

*Schistosoma japonicum* eggs can release miRNA-containing EVs, which can transfer their cargo miRNAs to recipient cells in vitro. Moreover, egg EVs associated miRNAs (i.e. Sja-miR-71b and Sja-bantam) were detectable in the primary hepatocytes of mice infected *S. japonicum*. Further work is required to identify the protein components within *S. japonicum* egg EVs and to understand whether and how each of these cargo molecules contribute to the fundamental interactions.
between the parasite and its host. Further exploration of the functions of these vesicles may help identify novel strategies for control of schistosomiasis.

Additional files

Additional file 1: Figure S1. S. japonicum eggs isolated from rabbits infected with S. japonicum cercariae 44 dpi. (TIF 6650 kb)

Additional file 2: Table S1. The list of schistosome small RNAs (over 100 reads) associated with S. japonicum egg extracellular vesicles (EVs). (XLSX 126 kb)

Abbreviations

DAPI: 4′,6-diamidino-2-phenylindole; dpi: days post-infection; EVs: Extracellular vesicles; miRNAs: microRNAs; PAGE: Polyacrylamide gel electrophoresis; qRT-PCR: quantitative real-time PCR; snRNA: small non-coding RNA; TEM: Transmission electron microscopy

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and the additional files. The newly-generated miRNA sequence (miR7) was deposited in the GenBank database under the accession number KY021901.

Authors’ contributions

SZ and WP conceived and designed the study. SZ, SW, YL, PJ, XC, XW and KY021901 contributed to the acquisition of the data. SZ performed the experiments and analyzed the data. SZ and WP wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study was carried out in strict accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals of the State Science and Technology Commission. All animal studies and protocols were approved by the Internal Review Board of Tongji University School of Medicine (permit number: TJLAC-015-028).

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References

1. Colley DG, Busidnyu AL, Secor WE, King CH. Human schistosomiasis. Lancet. 2014;383:2253–64. doi:10.1016/S0140-6736(13)61949-2.
2. Yang GJ, Sun LP, Hong QB, Zhu HR, Yang K, Gao Q, Zhou XN. Optimizing molluscicide treatment strategies in different control stages of schistosomiasis in the People’s Republic of China. Parasit Vectors. 2012;5:260. doi:10.1186/1756-3305-5-260.
3. Zhou XN, Wang LY, Chen MG, Wu XH, Jiang QW, Chen Y, et al. The public health significance and control of schistosomiasis in China - then and now. Acta Trop. 2005;96:97–105.
4. Zhou YB, Liang S, Jiang QW. Factors impacting on progress towards elimination of transmission of schistosomiasis japonica in China. Parasit Vectors. 2012;5:275. doi:10.1186/1756-3305-5-275.
5. Zhu L, Liu J, Cheng G. Role of microRNAs in schistosomes and schistosomiasis. Front Cell Infect Microbiol. 2014;4:165. doi:10.3389/fcimb.2014.00165.
6. Driguez P, Mcmanus DP, Gobert GN. Clinical implications of recent findings in schistosome proteomics. Expert Rev Proteomics. 2016;13:19–33. doi:10.1586/14789450.2016.116390.
7. Zhu J, Xu Z, Chen X, Zhou S, Zhang W, Chi Y, et al. Parasitic antigens alter macrophage polarization during Schistosoma japonicum infection in mice. Parasit Vectors. 2014;7:122. doi:10.1186/1756-3305-7-122.
8. Zhu L, Dao J, Xi X, Li H, Lu K, Liu J, et al. Altered levels of circulating miRNAs are associated Schistosoma japonicum infection in mice. Parasit Vectors. 2015;8:196. doi:10.1186/s13071-015-0686-5.
9. Mathivanan S, Fahnert CJ, Reid GE, Simpson RJ. Exocarta 2012: Database of exosomal proteins, RNA and lipids. Nucleic Acids Res. 2012;40:D1241–4. doi:10.1093/nar/gkr828.
10. Théry C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. Nat Rev Immunol. 2002;2:569–79.
11. Conde-Vancells J, Rodríguez-Suárez E, Embade N, Gil D, Matthiesen R, Valle M, et al. Characterization and comprehensive proteome profiling of exosomes secreted by Hepatocytes. J Proteome Res. 2008;7:5157–66.
12. Simpson RJ, Lim JW, Moritz RL, Mathivanan S. Exosomes: proteomic insights and diagnostic potential. Expert Rev Proteomics. 2009;6:267–83. doi:10.1586/14789450.2008.1116390.
13. Mittelbrunn M, Sánchez-madrid F. Intercellular communication: diverse structures for exchange of genetic information. Nat Rev Mol Cell Biol. 2012;13:328–35. doi:10.1038/nrm3335.
14. Théry C. Exosomes: secreted vesicles and intercellular communications. F1000 Biol Rep. 2011;3:15. doi:10.3402/f1000rep.163-15.
15. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9:654–9.
16. Li J, Liu K, Liu Y, Xu Y, Zhang F, Yang H, et al. Exosomes mediate the cell-to-cell transmission of IFN-γ-induced antiviral activity. Nature Immunol. 2013;14:793–803. doi:10.1038/ni.2647.
17. Mittelbrunn M, Gutiérrez-vázquez C, Villaroya-beltri C, González S, Sánchez-caboo F, González MA, et al. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. Nat Commun. 2011;2:282. doi:10.1038/ncomms1285.
18. Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan ML, Karlsson JM, et al. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood. 2012;119:756–66. doi:10.1182/blood-2011-02-338004.
19. Meckes Jr DG, Shair KH, Marquitz AR, Kung CP, Edwards RH, Raab-Traub N. Human tumor virus utilizes exosomes for intercellular communication. Proc Nat Acad Sci USA. 2010;107:20370–5. doi:10.1073/pnas.1014091107.

20. Pegtel DM, Cosmopoulos K, Thorley-Lawson DA, van Eijndhoven MA, Hopmans ES, Lindenberg JL, et al. Functional delivery of viral miRNAs via exosomes. Proc Natl Acad Sci USA. 2010;107:6328–33. doi:10.1073/pnas.0914843107.

21. Zhang L, Hou D, Chen X, Li D, Zhu L, Zhang Y, et al. Exogenous plant MIR168a specifically targets mammalian DDQR1. Evidence of cross-kingdom regulation by microRNA. Cell Res. 2012;22:107–26.

22. Oliveira DL, Freire-de-lima CG, Nosanchuk JD, Casadevall A, Rodrigues ML, Nimrichter L. Extracellular vesicles from Cryptococcus neoformans modulate macrophage functions. Infect Immun. 2010;78(4):1601–9. doi:10.1128/IAI.09171-09.

23. Koeppe K, Hampton TH, Jareck M, Scharf M, Gerber SA, Mielcarew DW, et al. A novel mechanism of host-pathogen interaction through sRNA in bacterial outer membrane vesicles. PLoS Pathog. 2011;7:e1002404.

24. Buck AH, Coakley G, Simbari F, McSorley HJ, Quintana JF, Le Bihan T, et al. Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity. Nat Commun. 2014;5:4588. doi:10.1038/ncomms4588.

25. Marcilla A, Trelis M, Cortés A, Scoño J, Cantalapiedra F, Minguet MT, et al. Extracellular vesicles from parasitic helminths contain specific excretory/secretory proteins and are internalized in intestinal host cells. Plos One. 2012;7:e45974. doi:10.1371/journal.pone.0045974.

26. Bernal D, Trelis M, Montaner S, Cantalapiedra F, Galán O, Hakkenberg M, Marcilla A. Surface analysis of Dicrocoelium dendriticum. The molecular
characterization of exosomes reveals the presence of miRNAs. J Proteomics. 2014;105:232–41. doi:10.1016/j.jprot.2014.02.012.

27. Zamanian M, Fraser LM, Agbedanu PN, Harischandra H, Moorhead AR, Day TA, et al. Release of small RNA-containing exosome-like vesicles from the human filarial parasite Brugia malayi. PLoS Negl Trop Dis. 2015;9:e0004069. doi:10.1371/journal.pntd.0004069.

28. Lambertz U, Oviedo Ovando ME, Vasconcelos EJ, Utrau PJ, Myler PJ, Reiner NE. Small RNAs derived from tRNAs and rRNAs are highly enriched in exosomes from both old and new world Schistosoma providing evidence for conserved exosomal RNA Packaging. BMC Genomics. 2015;16:15. doi:10.1186/s12864-015-1260-7.

29. Silverman JM, Clos J, De Oliveira CC, Shirvani O, Fang Y, Wang C, et al. An exosome-based secretion pathway is responsible for protein export from Leishmania and communication with macrophages. J Cell Sci. 2010;123:842–52. doi:10.1242/jcs.056465.

30. Nowacki FC, Swain MT, Klychnikov OI, Niazi U, Ivens A, Quintana JF, et al. Protein and small non-coding RNA-enriched extracellular vesicles are released by the pathogenic blood fluke Schistosoma mansoni. J Extracell Vesicles. 2015;4:28665. doi:10.3402/jve.v4.28665.

31. Sciotello J, Pearson M, Potriquet J, Becker L, Pickering D, Mulvenna J, et al. Extracellular vesicles secreted by Schistosoma mansoni contain protein vaccine candidates. Int J Parasitol. 2016;461–5. doi:10.1016/j.ijpara.2015.09.002.

32. Wang L, Li Z, Shen J, Liu J, Liang J, Wu X, et al. Exosome-like vesicles derived by Schistosoma japonicum adult worms mediates M1 type immune-activity of macrophage. Parasitol Res. 2015;114:1865–73. doi:10.1007/s00436-015-4377-7.

33. Zhu L, Liu J, Dao J, Li K, Li H, Gu H, et al. Molecular characterization of S. japonicum exosome-like vesicles reveals their regulatory roles in parasite-host interactions. Sci Rep. 2016;6:25885. doi:10.1038/srep25885.

34. Cai P, Piao X, Hao L, Liu S, Hou N, Wang H, Chen Q. A deep analysis of the small non-coding RNA population in Schistosoma japonicum eggs. PloS One. 2013;8:e40033. doi:10.1371/journal.pone.0040033.

35. Xue X, Sun J, Zhang Q, Wang Z, Huang Y, Pan W. Identification and characterization of novel microRNAs from Schistosoma japonicum. Plos One. 2008;3:e4034. doi:10.1371/journal.pone.0004034.

36. Sohn W, Kim J, Kang SH, Yang SR, Cho JY, Cho HC, et al. Serum exosomal microRNAs as novel biomarkers for hepatocellular carcinoma. Exp Mol Med. 2015;47:e184. doi:10.1038/emm.2015.68.

37. Hazan-Halevy J, Rosenblum D, Weinstein S, Bairey O, Raanani P, Peer D. Cell-specific uptake of mantle cell lymphoma-derived exosomes by malignant and non-malignant B-lymphocytes. Cancer Lett. 2015;364:59–69. doi:10.1016/j.canlet.2015.04.026.

38. He X, Sai X, Chen C, Zhang Y, Xu X, Zhang D, Pan W. Host serum miR-223 is a potential new biomarker for Schistosoma japonicum infection and the response to chemotherapy. Parasit Vectors. 2013;6:272. doi:10.1186/1756-3305-6-272.

39. Cheng G, Luo R, Hu C, Cao J, Jin Y. Deep sequencing-based identification of pathogen-specific microRNAs in the plasma of rabbits infected with Schistosoma japonicum. Parasitology. 2013;140(14):1751–61. doi:10.1017/S0031182013000917.

40. Marcilla A, Martin-Jaular L, Trelis M, de Menezes-Neto A, Ivens A, Quintana JF, et al. Extracellular vesicles in parasitic diseases. J Extracell Vesicles. 2014;3:25040. doi:10.3402/jve.v3.25040.

41. Cai P, Gobert GN, You H, Duke M, McManus DP. Circulating miRNAs: potential novel biomarkers for hepatopathology progression and diagnosis of schistosomiasis japonica in two murine models. PLoS Negl Trop Dis. 2015;9:e0003965. doi:10.1371/journal.pntd.0003965.

42. Mitchell PS, Parkin RK, Koh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci USA. 2008;105:10513–8. doi:10.1073/pnas.0804549105.

43. Villarroya-Beltri C, Gutierrez-Vazquez C, Sanchez-Cabo F, Perez-Hernandez D, Vazquez J, Martin-Coloretes N, et al. SUMOylated HmRNP2/B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. Nat Commun. 2013;4:2980. doi:10.1038/ncomms3980.

44. Ohshima K, Inoue K, Fujiiwara A, Hatakeyama K, Kanto K, Watanabe Y, et al. Let-7 microRNA family is selectively sequestered into the extracellular environment via exosomes in a metastatic gastric cancer cell line. Plos One. 2012;5:e13247. doi:10.1371/journal.pone.0013247.