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Communication

An evidence for a novel antiviral mechanism of teleost fish: serum-derived exosomes inhibit virus replication through incorporating Mx1 protein

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Abstract: Exosomes are associated with cancer progression, pregnancy, cardiovascular diseases, central nervous system–related diseases, immune responses and viral pathogenicity. However, study on the role of exosomes in the immune response of teleost fish, especially antiviral immunity, is limited. Herein, serum-derived exosomes from mandarin fish were used to investigate antiviral effect for the exosomes of teleost fish. Exosomes were isolated from mandarin fish serum by ultracentrifugation could internalize into Mandarin fish fry (MFF-1) cells and inhibited Infectious spleen and kidney necrosis virus (ISKNV) infection. To further investigated the underlying mechanisms of exosomes in inhibiting ISKNV infection. The protein composition of serum-derived exosomes was by analysis mass spectrometry and found that myxovirus resistance 1 (Mx1) was incorporated in the exosomes. Furthermore, the scMx1 protein was proved transferred to the recipient cells though the exosomes. Our results found that the serum-derived exosomes from mandarin fish could inhibit ISKNV replication and suggested an underlying mechanism of the serum-derived exosomes antivirus is that serum-derived exosomes incorporation of the Mx1 protein into exosomes and delivery into recipient cells. This study provided an evidence for the important antiviral role of exosomes in the immune system of teleost fish.

Keywords: Teleost fish, Exosome, Immunity, Mx1, ISKNV.

1. Introduction

Exosomes are extracellular vesicles of endosomal origin and have varied size, ranging from ~40 nm to 160 nm in diameter [1]. Many cell types release exosomes into the extracellular environment and are found in biological fluids, such as Semen, amniotic fluid, breast milk and blood [2]. Serum-derived exosomes are the most abundant and widely distributed in the body [3]. previous studies showed that serum-derived exosomes take part in numerous processes of organisms and the utility for the diseases diagnosis and therapy development [4, 5].

A wide range of physiological and pathological functions of exosomes were confirmed and attributed to transferring functional cargos [2]. Proteins, metabolites, and nucleic acids delivered by exosomes into recipient cells effectively alter their biological response [6, 7]. In mammals, exosomes are involved in multiple biological and pathological processes, such as reproduction and development, cancer progression, cardiovascular diseases, and immune responses, especially in virus infections [2]. Exosomes improve viral infectivity by spreading viral and cellular components, resulting in virus immune evasion and depressing the immune response [8]. Moreover, exosome direct exchange of interferon-stimulated genes, proteins, or mRNA transferred by exosome among host cells

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might contribute to the establishment of anti-viral state in recipient cells [9]. For example, Human macrophages shed exosomes that deliver antiviral mediators, including the single-stranded DNA cytidine deaminase APOBEC3G (apolipoprotein B mRNA editing enzyme, catalytic polypeptide like 3G), protecting human hepatocytes from HBV (hepatitis B virus) infection [10]; miR-1975 was secreted in exosomes and taken up by the neighboring cells to induce interferon expression suppressing influenza virus replication [11]. However, study on the role of exosomes in the immune response of teleost fish, especially antiviral immunity, is limited.

Although fish aquaculture is the fastest growing animal food sector to support the growing human population, fish production is threatened by numerous diseases [12]. Mandarin fish (Siniperca chuatsi) is an economically important species that is widely distributed in China; their industry is threatened by different aquatic viruses, e.g., Infectious spleen and kidney necrosis virus (ISKNV) and siniperca chuatsi rhabdovirus (SCRV) [13]. Thus, the antiviral mechanism of mandarin fish should be elucidated to prevent virus disease outbreak. In this study, serum-derived exosomes from mandarin fish were used to investigate antiviral effect for the exosomes of teleost fish.

2. Results

2.1. Exosomes were isolated from mandarin fish serum

In the current study, exosomes were isolated from mandarin fish serum by ultracentrifugation (Figure 1A). The purified exosomes were further identified by western blot analysis using the antibodies of three representative exosome markers [6], lysosomal associated membrane protein 3 (CD63), tumor suppressor gene 101 (TSG101), and heat shock 70 kDa protein-8 (HspA8) (Figure 1B). Transmission electron microscopy images showed that exosomes have a cup-shaped bilayer-enclosed morphology, ranging from 30 nm to 150 nm in size (Figure 1C). Furthermore, whether exosomes could internalize into mandarin fish fry (MFF-1) cells were investigated. Exosomes were labeled with green fluorescent dye PKH67 [14]. PKH67-labeled exosomes were incubated with cells for 2 hours, and green fluorescence was detected around the nucleus in MFFF-1 cells (Figure 1D). These results indicated that serum-derived exosomes from mandarin fish could internalize into recipient cells.

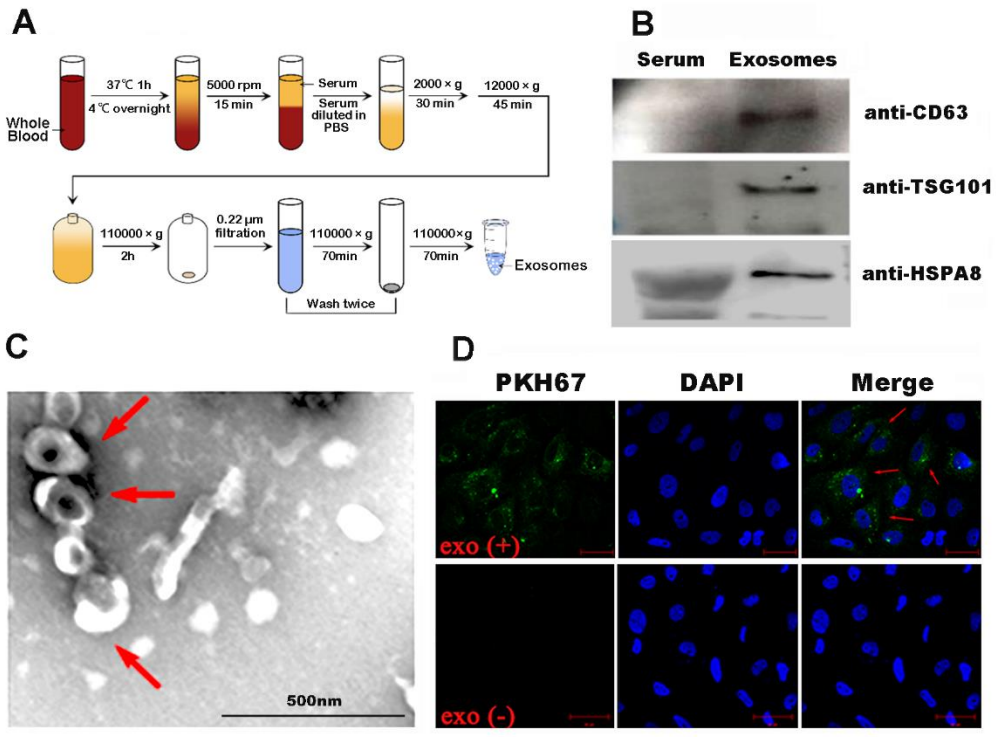

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Figure 1. Isolated exosomes from mandarin fish serum. (A) Schematic of the generation of purified serum-derived exosomes from mandarin fish by using differential centrifugation. (B) Purified exosomes (right) from mandarin fish serum were analyzed on Western blot with antibody directed against CD63, TSG101, and HspA8. The normal untreated mandarin fish serum (left) was used as control. (C) Transmission electron microscopy observations of the purified exosomes from serum. (D) Confocal microscopy assay showed that the exosomes were internalized by MFF-1 cells after incubation with PKH67-labeled exosomes for 2 h.

2.2. Serum-derived exosomes from mandarin fish inhibited ISKNV infection

The cells were co-cultivated with exosomes (0, 1, 5, 10 μg/mL) after ISKNV infection to investigate whether exosomes exert their antiviral function against fish virus. Viral levels were measured via qPCR and Western blot analyses at 48 h post-infection. The DNA level of the isknv-mcp gene was used to represent the level of the viral genome. As shown in Figure 1E, the DNA level of isknv-mcp obviously decreased after incubation with exosomes in a dose-dependent manner. Similar results were also observed in the levels of the ISKNV VP101L protein (Figure 1F), which is an important structural protein of ISKNV [15]. These results suggested that serum-derived exosomes from mandarin fish strongly inhibited ISKNV infection.

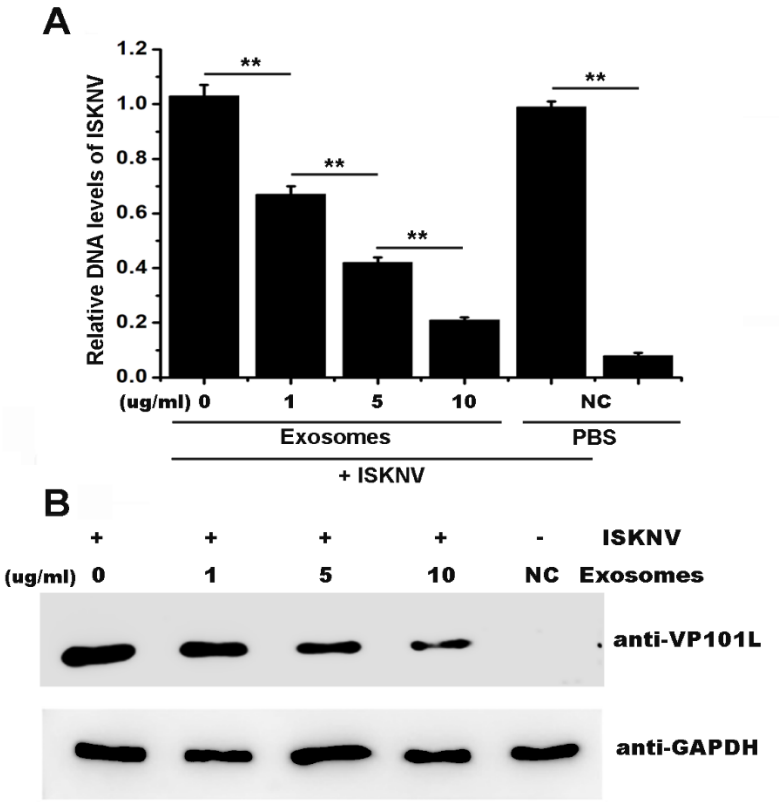

Figure 2. Mandarin fish serum-derived exosomes inhibite ISKNV infection (A) Cells were incubated with 0, 1, 5, and 10 μg/mL exosomes after infection with ISKNV at a MOI of 10 (lanes 1-4); Incubation with PBS as positive control (lane 5) and uninfected cells (lane 6) as negative control (NC); Vertical bars represent statistical significance as indicated by asterisks, with * referring to $p < 0.05$ and ** referring to $p < 0.01$. DNA levels of isknv-mcp were quantified via qPCR in MFF-1 cells at 48 h post-infection. β-actin served as a reference gene to calibrate the cDNA template for all samples. (B) Western blot analysis of the ISKNV-VP101L protein after 48 h of infection.

2.3 Mx1 protein was incorporated into the serum-derived exosomes.

The protein composition of serum-derived exosomes was investigated to further reveal the underlying mechanisms of exosomes in inhibiting ISKNV infection. Myxovirus resistance 1 (Mx1), a critical antivirus component of innate immunity induced by type I

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IFNs [16], was found in the exosomes by mass spectrometry analysis (Figure 1G), and was then identified by Western blot analysis using anti-Mx1 antibody (Figure 1F). The exosomes were treated with trypsin digestion to further verify the localization of the Mx1 protein in the exosomes. As shown in Figure 1H, the Mx1 protein was also detected when the exosomes were treated with trypsin, whereas the Mx1 protein was reduced in the exosomes treated with Trion X-100 before trypsin. These observations indicated that the Mx1 protein was incorporated into the serum-derived exosomes.

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A

Identification of serum exsomal proteins from mandarin fish by mass

Number	protein name	protein MW(kD)	protein pI	protein score
1	Alpha-enolase	47168.41	7.01	105.48
2	Phosphoglycerate kinase	17049.79	5.47	71.03
3	Glyceraldehyde-3-phosphate dehydrogenase	36052.79	8.57	52.98
4	Beta actin	41722.27	5.29	49.76
5	Flotillin-1	28165.06	8.25	29.75
6	Transcription elongation factor SPT6	199070.42	4.81	36.43
7	ADM2	15865.14	11.81	42.08
8	Acetyl-CoA carboxylase 2	12480.06	5.84	34.7
9	Interferon-induced GTP-binding protein Mx1	75519.44	5.6	29.42
10	Liprin-alpha-4	134039.53	5.86	26.4
11	Regulator of G-protein-signaling 9	57387.04	9.32	21.77
12	TBC1D1 protein	102876.32	6.08	27.2
13	Transmembrane protease serine 11B	46336.68	9.04	30.1
14	Nesprin-2	787714.06	5.33	35.2
15	Thyroid peroxidase	40686.25	6.25	30.4
16	Plexin-A3	207700.53	7.06	21.49
17	Tyrosine kinase	7486.32	4.6	23.55
18	Protein AHNAK2	616622.3	5.2	22.54
19	Vacuolar protein sorting-associated protein 37	38658.49	5.19	22.57
20	Piwi-like protein	98602.07	9.5	21.79

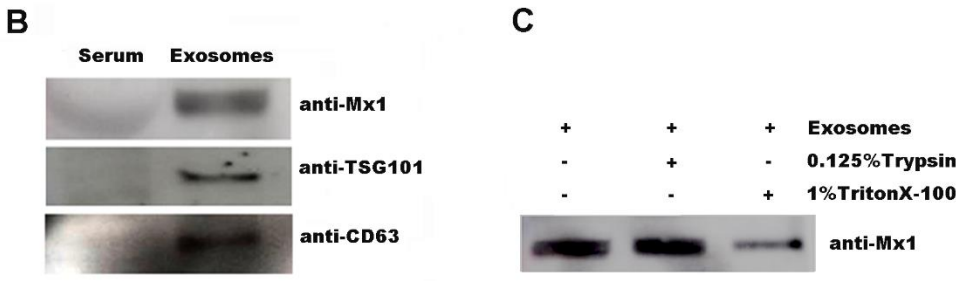

Figure 3. The protein composition of mandarin fish serum-derived exosomes.(A) Purified serum-derived exosomes from mandarin fish were analyzed with LC-MS/MS to determine the host proteins. (B) Mx1 proteins presented in serum-derived exosomes were confirmed on Western blots with antibody directed against Mx1. Left: untreated mandarin fish serum. Right: purified serum-derived exosomes. (C) The assay of exosomes resistance to trypsin digestion. The same amounts of exosomes were used for each condition (10 μg); the line 1 is control, mock treated (exo); the line 2 is incubated with trypsin (0.125% Trypsin) for 30 min at room temperature; the line 3 is treated with 1% Triton X-100 for 5 min before incubation with trypsin. Western blot analysis with antibody directed against Mx1.

2.4 Mx1 protein could be transferred to the recipient cells though the exosomes.

The Mx1-GFP-tag plasmid was constructed and transfected into MFF-1 cells to further confirm whether Mx1 could be incorporated into cellular exosomes. At 24 h post-transfection, exosomes were isolated from the cell culture supernatant and then analyzed by Western bolt. The results showed that the Mx1-GFP recombinant protein was detected in the cellular exosomes (Figure 1J), indicating that this protein was packaged into the exosomes. In addition, Mx1-GFP-labeled cellular exosomes could rapidly endocytosed

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into MFF-1 cells and colocalized with markers specific for late endosomes/lysosomes (Figure 1K). These results suggested that the Mx1 protein could be transferred to the recipient cells though the exosomes.

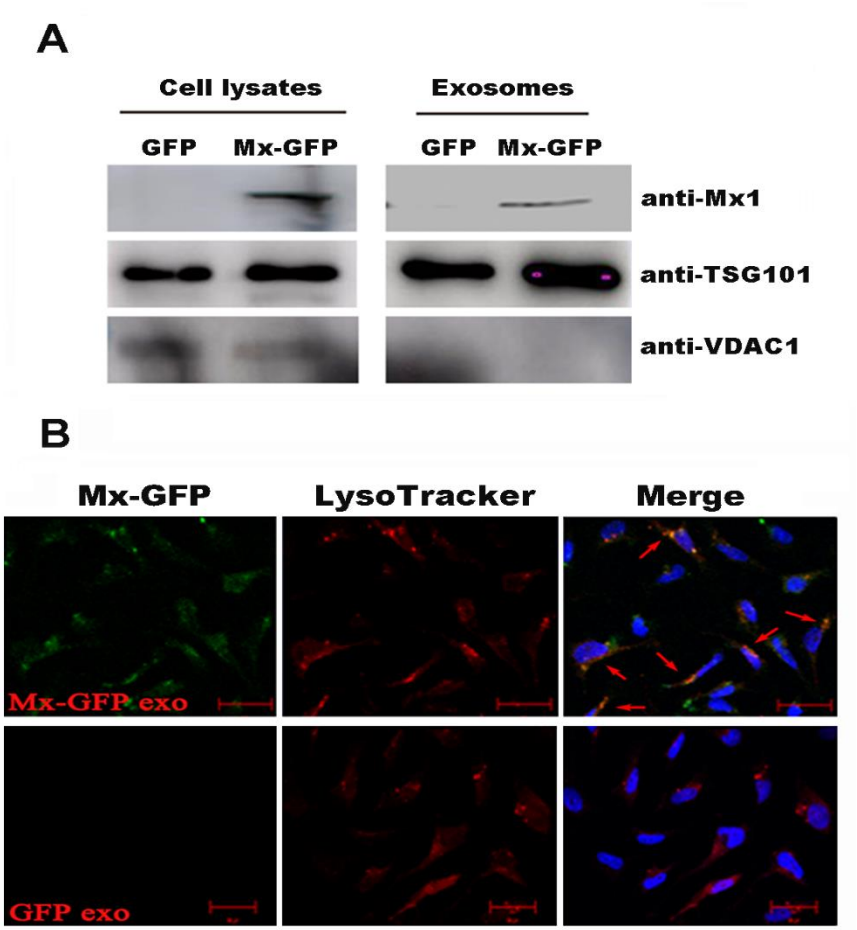

Figure 3. Mx1 protein transferred to the recipient cells though the exosomes.(A) Cells were transfected with Mx1-GFP-expressing vectors secreted exosomes with Mx1. Exosomes were isolated from cell cultures from transiently transfected MFF-1 cells. Cellular and exosomal lysates were analyzed by Western blot for the presence of Mx1, TSG101, and Voltage-dependent anion channel protein 1 (VDAC1, a mitochondrial marker). (B) Confocal microscopy assay showed that the Mx1-GFP exosomes were internalized by MFF-1 cells. The cells were transfected with Mx1-GFP-expressing vector, and the exosomes were isolated from culture supernatant. Exosomes were incubated with MFF-1 cells for 2 h and then with LysoTracker (LT) for 1 h.

3. Discussion

Extracellular vesicles (EVs) circulate in the body fluids of all organisms where they participate in intercellular cross-organ communication [17]. Exosomes are EVs with a size range of ~40 to 160 nm (average ~100 nm) in diameter with an endosomal origin. The exosomes were identified in fish such as rainbow trout, tongue sole and zebra fish [18-20]. In present study, the exosomes were firstly isolated from mandarin fish serum by ultracentrifugation. Exosomes are associated with immune responses has been widely documented [21, 22]. Recent experiments show that exosomes help to prevent infection such as poliovirus, human cytomegalovirus, and herpes simplex virus 1 [23]. In fish, the exosomes take part in A variety of physiological processes such as sex maturation and stress response [19, 24]. However, the role of teleost fish exosomes in antiviral effect, is limited.

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The function of serum-derived exosomes was also investigated in this work. the serum-derived mandarin fish exosomes could internalize into MFF-1 cells and inhibited ISKNV replication indicated that the teleost fish exosomes also play a role in anti-viral effect.

The function of exosomes in immune regulation is likely due to the transfer and presentation of antigenic peptides, delivery of signaling molecule to recipient cells, gene-expression manipulation by exosomal miRNA, and induction of different signaling pathways by surface ligands present on the exosomes [2]. In present study, the protein composition of serum-derived exosomes was analyzed by LC-MALDI MS/MS and identified Mx1 protein from the exosomes. Mx1 is recommended as interferon-induced GTP-binding protein, belonged to the dynamin superfamily of large GTPases that associate with intracellular membranes and are involved in a wide range of intracellular transport processes [25]. It was a critical components of innate immunity while most Mx genes are induced by type I IFNs in response to viral infection. Most of Mx family members have a specificity antiviral profiles against number of RNA and DNA viruses [16]. Furthermore, the localization of Mx1 protein in exosomes were identified by trypsin digestion and found that Mx1 was not on the surface of the exosomes, but incorporated into the exosomes. The Mx1-GFP-labeled cellular exosomes were endocytosed into MFF-1 cells proved that Mx1 protein could be transferred to the recipient cells though the exosomes. Those findings suggested that the anti-viral effect of this serum-derived exosome is directly delivery the Mx1 into the recipient cells to suppress ISKNV replication.

In conclusion, our results suggested that the serum-derived exosomes from mandarin fish could inhibit ISKNV replication, of which the underlying mechanism of the antivirus function might be the incorporation of the Mx1 protein into exosomes and delivery into recipient cells. This study provided an evidence for the important antiviral role of exosomes in the immune system of teleost fish.

4. Materials and Methods

4.1. Cells, virus, and animals.

Mandarin fish fry (MFF-1) cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 27°C in 5% CO2 [26]. The ISKNV used in this study was originally isolated from disease-infected mandarin fish and preserved in our laboratory. Mandarin fish samples (500g) were obtained from the fish farms in Foshan, Guangdong, China. All animal experiments were performed in accordance with the regulation for animal experimentation of Guangdong Province, China and were allowed by the ethics committee of Sun Yat-sen University.

4.2. Exosomes isolation from fish serum and electron microscopy analysis.

Collected fresh blood from caudal vein of healthy mandarin fish in the sterile tube, incubated at 37°C for 1 h and incubated at 4°C overnight. Then the samples were centrifugation at 5000 rpm for 15min at 4°C and collected the supernatant serum. Diluted the serum with phosphate buffer solution (PBS) at the ratio of 1:10 before centrifugate at 2000×g for 30min at 4°C. Next, centrifugate the supernatant at 12,000 ×g for 45min at 4°C. Transfer the pre-cleared supernatant to a SW40 pipe and ultracentrifugation at 110,000 ×g for 2 h at 4°C (SW40, Beckman). After that, the precipitate was resuspended in a large volume of PBS and passed through a 0.22 mm pore PES filter (Millipore). Then this supernatant (pre-cleared medium) was next followed by ultracentrifugation 110,000 ×g for 70 min at 4°C (SW40, Beckman) to sediment exosomes. The precipitate was resuspended with PBS and subjected to the same ultracentrifugation conditions to wash the sample. The precipitate was resuspended with PBS, stored at 4°C in short period while -80°C in long-term. Exosomes shapes were observed through transmission electron microscopy (TEM) after purification [27].

4.3. Western blot analysis.

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Cells and exosomes were collected, washed twice with ice-cold PBS, and solubilized in Cell lysis buffer for Western and IP (Beyotime) were then centrifuged at 10,000 ×g at 4°C to remove cellular debris. Protein concentrations of the samples were determined using Bicinchoninic acid (BCA) protein assay kits (Beyotime). The purified samples (20 μg) were mixed with 5× loading buffer (250 mM Tris-HCl pH 6.8, 10% SDS, 5% β-mercaptoethanol, 50% glycerinum, 0.5% bromophenol blue), boiled for 10 min, and then subjected to SDS-PAGE for separation. Western blot analysis was performed as described previously. Tsg101, Mx1, HspA8, VDAC1, GAPDH antibodies were purchased from Abcam (UK). GFP-tag antibodies were obtained from Proteintech (Wuhan, China). ISKNV-ORF101 and sc-CD63 antibodies were prepared for this experiment.

4.4. Exosome infection assays

Cells were seeded on 24-well plates at confluent of 70% and incubated with different concentrations of exosomes for 12 h. Then removed the media slightly and infected with ISKNV with a multiplicity of infection (MOI) of 1 and incubated for 2 h at 27°C. And then washed off the unbound virus thrice and replaced the medium with fresh maintenance medium at the same concentrations of exosomes. At 48 h post-infection, cell samples were collected for further analysis.

4.5. Quantification of Viral DNA by qPCR

ISKNV genome was determined using a LightCycler480 instrument (Roche, Germany). qPCR was performed using isknv-mcp gene primers, forward (isnknv-mcp F primer 5′- CAATGTAGCACCCGCACTGACC -3′) and reverse (isnknv-mcp R primer 5′- ACCTCACGCTCCTCACTTGTC-3′); β-actin served as a reference gene to calibrate the cDNA template, forward (β-actin F primer 5′- CCCTCTGAACCCCAAAGCCA-3′) and reverse (β-actin R primer 5′- CAGCCTGGATGGCAACGTACA -3′) primers. DNA from the infected cells was extracted using DNeasy Blood & Tissue Kits (Qiagen, Germany). The extracted DNA template were subjected to qPCR. The PCR reaction mixture (10 μl) contained 5 μl 2 × SYBR Premix Ex Taq (TaKaRa, China), 1 μl DNA template, 0.2 μl of 10 μM primers, and 3.6 μl H2O. The qPCR conditions were as follows: one cycle at 95 °C for 10 s, 40 cycles of 5 s at 95 °C, 40 s at 60 °C, and 1 s at 72 °C. qPCR was performed at three replicates per sample.

4.6. Proteomics and data analysis

Exosomes isolated from mandarin fish serum were harvested and lysed. After the protein concentration was detected with the BCA assay. Peptides obtained after digestion were subjected to LC-MALDI MS/MS analysis in the Research Center for Proteome Analysis (Shanghai, China). Finally, the proteins were analyzed with Triple TOP DDA or SWATH.

4.7. Confocal microscopy assay

Cells were grown at low density on 24-well plates with glass coverslips. Cells incubated with PKH67 (sigma, USA) marked exosomes for 2 h. And then incubated with Hoechst 33342 (Invitrogen, USA). Images were taken with a confocal microscope (Zeiss LSMS7 DUO NLO, Germany). Cells were grown at low density on 24-well plates with glass coverslips. Cells incubated with Mx1-GFP-tag exosomes for 2 h. And then replaced the media with exosomes-free media contain 100 nm Lysotracker Red DND-99 (Invitrogen, USA). After incubated for 1h, cells were fixed with 4% paraformaldehyde for 15 min at room temperature (RT). After washing with PBS three times, the coverslips were blocked with 5% normal goat serum solution for 30min. The coverslips were then washed several times with phosphate-buffered saline with Tween 20 and incubated with Hoechst 33342 (Invitrogen, USA). Images were taken with a confocal microscope (Zeiss LSMS7 DUO NLO, Germany). Mock-incubated cells were similarly stained as controls.

4.8. Plasmid construction and transient transfection.

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Recombinant DNA techniques were performed according to standard procedures. The primers used in this study were forward (pEGFP-N3-scMx-F primer 5′-CAATGTAG-CACCCGCACTGACC-3′) and reverse (pEGFP-N3-scMx-R primer 5′-ACCTCAC-GCTCCTCGCTTGTC-3′) primers. Mandarin fish Mx-1 DNA sequence from MFF-1 cells was cloned pEGFP-N3 to generate scMx1-GFP. The transient transfection of recombinant DNA plasmids into MFF-1 cells was performed using FuGENER HD Transfection Reagent ® (Promega, USA) according to the instructions of the manufacturer.

4.9. Exosomes isolation from transfection cultured cells.

For exosome isolation, MFF-1 cells grown in 150 mm cell culture dishes were washed thrice with PBS at approximately 80% confluence and cultivated with medium with DMEM containing 10% FBS depleted of bovine serum extracellular vehicles (EVs) by overnight ultracentrifugation at 100,000 ×g at 4°C after transfected with plasmids. After 48 h, the conditioned medium (CM) was collected and was first pre-cleared by centrifugation at 300 ×g for 10min at RT to remove the floating cells. And then all following centrifugation steps were performed at 4°C.Next, the supernatant was spun at 20,000 ×g for 20min to remove dead cells and shedding vesicles. Then, to collect exosomes, the supernatant was isolated by ultracentrifugation at 110,000 ×g for 70min (Ti70, Beckman) and removed the supernatant. After that, the precipitate was resuspended in a large volume of PBS and passed through a 0.22 mm pore PES filter (Millipore). Then this supernatant (pre-cleared medium) was next followed by ultracentrifugation 110,000 ×g for 70min (SW40, Beckman) to sediment exosomes. The precipitate was resuspended with PBS and subjected to the same ultracentrifugation conditions to wash the sample.

Author Contributions: Chang-Jun Guo, and Jian-Guo He conceived the study. Jian He, and Shao-Ping Weng performed sample preparation of fish. Jian He, Zhi-Min Li, Nan-Nan Chen and Yuan-Yuan Wang performed the experiments. Jian He, and Chang-Jun Guo performed the picture drawing and processing of this article. Jian He, and Chang-Jun Guo wrote the manuscript. All authors discussed the results and commented on the manuscript.

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Institutional Review Board Statement: This study was approved by the Institutional Animal Care and Use Ethics Committee of Sun Yat-sen University.

Data Availability Statement: Data available in a publicly accessible repository.

Conflicts of Interest: The authors declare no conflict of interest.

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