Echinococcus Granulosus Extracellular Vesicles miRNA Cargo Regulates the Encystation of Protoscoleces via Notch Signaling Pathway in Vitro

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Research Article

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Abstract

Background: Since the protoscolece (PSCs) of *Echinococcus granulosus* has the characteristics of differentiation into hydatid in the intermediate host, it may be the main cause of secondary hydatid infection and dissemination. Extracellular vesicles (EVs) can transfer non-coding RNAs especially small RNAs (miRNAs) into parasite cells to regulate their gene expression. However, developmental pathway-related miRNAs such as the Notch signaling pathway loaded by EVs are not fully characterized. Therefore, the current study aimed to screen mRNA-miRNA interaction genes involved in the Notch signaling pathway during the encystation of *E. g* in vitro, and observe their expression changes in the parasite and extracellular vesicles (EVs).

Methods: The miRNA and mRNA profiles of PSCs and microcysts (MCs) of *E. g* were analyzed using high-throughput sequencing respectively. Differential mRNAs between PSCs and MCs were overlapped with mRNAs which were predicted from differential miRNAs between PSCs and MCs. Functions of candidate differential mRNAs were predicted using Gene Ontology enrichment, Kyoto Encyclopedia of Genes, and Genomes pathway analysis. The miRNA-mRNA subnetwork related to the Notch signaling pathway was established. The expression of Notch pathway-related miRNAs and its target mRNAs in the worms and EVs at different encystation time points by Quantitative Reverse Transcription–Polymerase Chain Reaction (qRT-PCR).

Results: 9439 mRNAs were predicted from 39 differential miRNAs between PSCs and MCs and overlapped with 1586 differential mRNAs in the transcriptome library, 1445 differential mRNAs were screened out and clustered into 94 metabolic pathways. 5 differential miRNAs corresponding to 4 target mRNAs were enriched in the Notch signaling pathway, especially containing a new unknown miRNA eg- m0694-3p. The 4 target mRNAs, EgrG_000892700, EgrG_001029400, EgrG_001081400 and EgrG_000465800 were respectively annotated as TACE, CSL, CtBP, and Delta in the Notch signaling pathway. EgrG_000892700, which was annotated as ADAM17 / TACE in the Notch signaling pathway, was down-regulated during encystation process of PSCs. Three miRNAs, egr-miR-4989-3p, egr-miR-9-3p and egr-miR-277a-3p might have negative regulatory effects on EgrG_000892700. The expressions of egr-miR-4989-3p and egr-miR-277a-3p in EVs of MCs were nearly 5 times that of PSCs.

Conclusions: EVs may negatively regulate the expression of EgrG_000892700 in worms through continuous targeting of egr-miR-4989-3p and egr-miR-277a-3p, inhibit the transcription of the Notch signaling pathway, and may participate in the regulation of encystation of *E. granulosus*.

Background

As one of the zoonotic disease caused by the larvae of *Echinococcus granulosus* (*E. granulosus, E. g*), Cystic echinococcosis (CE) shows a world-wide distribution and poses a serious threat to human health and socio-economic burden[1]. There are 5~30% of the population in western China is positive for *E. g*
antibody, indicating that a large number of individuals have been exposed to this parasite[2]. Prevention and treatment cannot be ignored.

Hydatid, as the pathogenic stage of CE, contains a large amount of Hydatid fluid(HF), daughter cysts and protoscoleces(PSCs) in the cyst wall. Since PSC have the developmental characteristics of differentiation into new hydatid cysts in the intermediate host, the dissemination and secondary infection of PSCs after the rupture of the cyst wall becomes the main reason for postoperative recurrence. Although the life cycle of *E. g* has been clearly studied, the main developmental mechanism is not completely clear, especially the regulation mechanism of non-coding RNA on the development of worms.

miRNA is a kind of endogenous non-coding RNA with a length of about 22 nucleotides[3], which has a significant effect on the molecular regulation mechanism of parasitic diseases[4]. miRNA is highly conservative, but there are differences in different developmental stages of organisms. Metachronic changes in expression and a small degree of spatial differences in expression make the expression of miRNAs dynamically regulated at different stages of cell development. From 2011 Cucher[5] first cloned 38 different *Eg* miRNAs, until 2020 Bai, Y[6] identified 167 known mature miRNAs or miRNA stars, high-throughput sequencing technology has made researches on miRNAs in full swing. More studies found that a variety of known and newly discovered *E. g* miRNAs have potential interactions with the five major signaling pathways of wnt, cAMP, Hedgehog, NF-kB and Notch, further confirming that miRNAs play an important regulatory role in the differentiation and development of *E. g*. The Notch signal transduction plays a key role in cell development and cell-cell communication in vertebrates and invertebrates, including the regulations of cell fate, migration, differentiation, and proliferation[7]. Studies related to the development and regulation of *Echinococcus granulosus* were mostly focused on the wnt pathway but rarely on the Notch signal pathway, which prompted for further exploration.

In addition, extracellular vesicles (EVs) encapsulated by lipid bilayers are released during the growth and development of eukaryotes, which play an important role in the cellular microenvironment, and are rich in nucleotides such as miRNA and mRNA[8]. Siles-Lucas[9] reported the exocrine body in the cyst fluid of sheep liver echinococcosis for the first time in 2017. Zhang[10] identified miRNA which may be related to host immunity and pathogenesis in the culture supernatant of PSCs and cyst fluid exocrine of *E. g*. These studies suggest that EVs as carriers of miRNAs may play an important role in different developmental stages.

In the present study, we found *E. g* PSCs continuously secrete EVs during the process of encystation in vitro. High throughput sequencing was conducted to analyze the mRNAs and miRNAs in protoscoleces(PSCs) and microcysts(MCs). The miRNA-mRNA subnetwork related to Notch signaling pathway were constructed. The expression levels of the above-mentioned mRNAs and miRNAs were verified in the worms and EVs during the process of encystation by qRT-PCR. The purpose of this study was to screen out the mRNAs and miRNAs involved in the regulation of the Notch signaling pathway during the encystation of *E. granulosus*, and to provide preliminary information for the EVs carring
miRNAs to participate in the regulation of the encystation of *E. granulosus*. The study might open up new ideas for blocking the secondary infection of *E.g* PSCs through EVs-miRNAs.

**Methods**

**Parasite Material and in vitro culture of PSCs of *E.g***

Liver hydatid cysts were collected aseptically from a slaughterhouse in Urumqi, China. The HF and PSCs were obtained from the fertile cysts under aseptic conditions and were placed in 50mL falcon tubes. The PSCs were washed 5–8 times using 0.9% NaCl containing 100 U/mL penicillin and 100µg/mL streptomycin (Gibco/Life Technologies, Carlsbad, CA, USA). The PSCs were purified once with aseptic 40-mesh stainless steel screen and 80-mesh stainless steel screen, then digested with aseptic pepsin solution with 1% pH value of 2~3, 30min in a water bath at 37 °C. The digested PSCs were washed repeatedly with 1% double anti-normal saline for 10 times, and finally re-suspended with the complete culture medium of RPMI-1640. The main components of the medium include RPMI-1640(Gibco/Life Technologies, Carlsbad, CA, USA), double antibodies(200 U/mL penicillin and 200µg/mL streptomycin), glucose (4 mg/mL) and 5% fetal bovine serum(Gibco/Life Technologies, Carlsbad, CA, USA), Fetal bovine serum was ultracentrifuged at 110,000g overnight to remove EVs in the serum. Viability was determined using a eosin stain dye exclusion test. The qualified PSCs(Survival rate ≥ 90%), re-suspended in the complete culture medium of RPMI-1640, were inoculated into T25 culture bottle at the density of 2 000 cells / mL, and cultured in 5% CO2 constant temperature incubator(Thermo Scientific Forma™ 310, USA) at 37 °C. The medium was changed every 2 to 3 days to establish a 90-day in vitro culture model of *E. g* PSCs, and the morphological changes of PSCs during the encystation were recorded under the inverted microscope digital imaging system(Nikon ECLIPSE Ts2R, Nikon, Japan). The encystation rate and the change of cyst diameters were calculated every five days, and the change curve were drawn. Average microencystation rate = (microcysts/total number of worms in the field of view)×100%. 10 PSCs in the field of view were randomly selected, the diameters of the PSCs or MCs were measured with a microscope micrometer, and the average diameter of the three fields of view was calculated.

**Library Preparation and Sequencing of mRNA and RNA-SEQ Analysis**

PSCs and MCs were cultured in vitro for more than 60 days were collected separately. The samples were added 1ml Trizol Reagent(Invitrogen, Frederick, MD, USA) and mixed thoroughly, -80°C degrees cryopreservation, Prepared for total RNA extraction. Total RNA was extracted through TaKaRa MiniBEST Universal RNA Extraction Kit(TaKaRa, Japan). The mRNA-Seq library(Non-strand-Specific) construction and sequencing were conducted by Personal Biotech Co., Ltd. (Nanjing, China). The quality, quantity, and integrity of the total RNAs were assessed using Thermo Scientific NanoDrop 2000(Thermo Scientific, Waltham,Massachusetts,USA) and an Agilent 2,100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively.
The mRNAs with polyA structure were enriched in total RNA by the Oligo (dT) magnetic bead method, and were broken into fragments of about 300 bp in length by ion interruption to facilitate the generation of clusters in the on-machine sequencing process. Using the mRNAs as a template, the first strand of cDNA is synthesized using a 6-base random primer and reverse transcriptase, and the second strand cDNA is synthesized using the first strand cDNA as a template. After completion of library construction, library fragments were enriched by PCR amplification, and the library of 450bp size was selected. The quality, total concentration and effective concentration of the library were detected by Agilent 2100 Bioanalyzer. The libraries containing different Index sequences were further mixed in proportion. The mixed library was uniformly diluted to 2nM, and the single-stranded library was formed by alkali denaturation. Finally, using Next-Generation Sequencing (NGS), based on the Illumina sequencing platform, paired-end (PE) sequencing was performed on these libraries.

After the 3'end with the linker sequence and the low-quality data were removed from the mRNAs raw data (Raw Data), the high-quality sequences were compared with the E.g reference genome (https://www.genedb.org/#/species/E. granulosus, E.granulosus_contigs.Fasta) through the HISAT2 software. FPKM, as the number of fragments from a certain gene per kilobase length per million fragments, was obtained by the HTSeq software from the Reads Count value of the aligned sequence number, and further calculated by the DESeq software to obtain the standardized average expression of mRNAs (baseMean) to make the expression levels of different genes comparable. The differentially expressed genes were screened by DESeq software, and the threshold set to identify differentially expressed mRNAs was |log2FoldChange|>1 and P<0.05.

**Small RNA Library Construction, Sequence Analysis, and Identification of miRNAs**

Small RNA were extracted through RNAiso for Small RNA Kit(TaKaRa, Japan). The small RNA library construction and sequencing were conducted by Personal Biotech Co., Ltd. (Nanjing, China). The small RNA library was established using NEB Next Multiplex Small RNA Library Prep Set for Illumina Kit (New England Biolabs Inc; Ipswich, Massachusetts, USA). Using total RNA as a raw material, small RNAs were efficiently enriched through the improvement and ligation of 3'-end and 5'-end joints. the first strand of cDNA was synthesized by reverse transcription, and the library fragments were enriched by PCR amplification. The PCR amplification products were recovered and purified by gel electrophoresis, and were tested for quality by Agilent 2100 Bioanalyzer and Agilent High Sensitivity DNA Kit. The library was quantified by fluorescence using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, California, USA). The effective library concentration was quantitatively detected by StepOnePlus Real-Time PCR Systems (Thermo Scientific, Waltham, Massachusetts, USA). Multi-sample DNA libraries (multiplexed DNA libraries) were homogenized and mixed in equal volumes. The mixed library was gradually diluted and quantified, and then sequenced in PE150 mode on the Illumina sequencing platform.

The miRNA raw data were saved in FASTQ format. High-quality data (Clean Reads) with a sequence length of 18 nt-36 nt were counted, and duplicate sequences were removed to obtain unique reads (Unique
Reads). The reference sequence of the miRNA precursor and mature body of *E. g* was obtained from the miRBase database. The comparison between Unique Reads and *E. g* genome sequence, the annotation of the obtained miRNAs, and the identification of conserved miRNAs were all statistically performed by Bowtie software. For unannotated sequences, unknown miRNAs were obtained by prediction of new miRNA precursors by mireap software. According to the number of sequences aligned to mature miRNAs of *E. g*, the number of sequences aligned to a gene (Read Count) was counted. If there were miRNAs with the same name in different locations, the abundance of the miRNA that appears for the first time was used for subsequent analysis, and the new miRNA sequence and expression level were predicted at the same time. The Reads Count value and the exon count per million mapped reads (CPM) were calculated by the DESeq software. The threshold set to identify differentially expressed miRNAs was fold change ≥2.0 and *P* < 0.05. Taking the 3'UTR sequence of the mRNAs of *E. g* as the target sequence, the target genes of the differential miRNAs were predicted by miRanda software.

**Bioinformatics analysis**

After the target mRNAs predicted by the differential miRNAs and the mRNAs obtained by actual transcriptome sequencing were crossed, candidate target mRNAs were screened. The GO enrichment analysis and KEGG enrichment analysis of candidate target mRNAs were calculated by topGO software and KAAS software respectively by the hypergeometric distribution method. *P* value < 0.05 were regarded as a statistically significant enrichment of differential mRNAs. Statistically significant GO terms were found, the pathways to which candidate target mRNAs been enriched were determined, and the biological functions of miRNAs related to the target genes were annotated. The target mRNAs enriched in the Notch signaling pathway were screened, and the subnetwork of miRNAs - mRNAs related to the Notch signaling pathway was drawn by Cytoscape software. According to the high-throughput sequencing, the differential expression content was abundant, and the number of connections with the target gene (degree) ≥ 3 meet the candidate criteria.

**TEM, NTA and Western bloting identification of extracellular vesicles (EVs)**

Corresponding to the three time points of encystation of PSCs in vitro, collection of EVs in the culture supernatant respectively occurred on day 3-10, day 55-62 and day 80-87 of culture in vitro. Before the collection of EVs, the worms were washed 3-5 times with sterile PBS and resuspended in serum-free medium. The parasite culture medium was harvested and changed every 12 h. After being centrifuged at to remove impurities, The PSCs culture mediums were successively centrifuged at 300×g for 10min, at 2,000×g for 20min, and finally at 10000×g for 40min to remove large dead cells and cell debris. The supernatants were filtered using low-protein binding 0.22 µm pore filters (Millipore, Bedford, MA, USA), then concentrated by Amicon Ultra-15 100 kDa ultrafiltration tube (Millipore, Bedford, MA, USA), centrifuged at 4°C, 4000×g for 10-20 min to collect all the concentrated liquid. The concentrated liquids were subjected to ultracentrifugation at 110000×g for 90min at 4°C to pellet the vesicles using a Beckman Coulter Optima L-100 XP ultracentrifuge (Beckman Coulter, Indianapolis, IN, USA), resuspended in 100 µL of PBS and stored at −80°C until use.
20 µL of the EVs suspension was dropped on the copper mesh of the electron microscope, dried under an incandescent lamp, negatively stained in a 2% uranyl acetate aqueous solution for 5 minutes, washed with pure water to remove excess dye solution, and dried with filter paper. After drying at room temperature, the sample was observed under a transmission electron microscope (G2 Spirit BioTWIN, Tecnai, Germany).

The EVs suspension is sufficiently mixed by pipetting and diluted 1000-fold. Before the samples were detected on the machine, the cuvette of the Nanoparticle Tracking Analyzer (ZetaView PMX 110, Particle Metrix, Meerbusch, Germany) was pre-washed with deionized water for more than three times and calibrated with polystyrene microspheres (110nm). The detection test results were processed by the software ZetaView 8.04.02 SP2.

The EVs samples were lysed on ice with RIPA lysis buffer (Abcam, USA) containing 1% protease inhibitor (PMSF) for 30 min, and then centrifuged at 10000×g and 4°C for 10 min. The protein sample concentrations were calculated by the BCA protein concentration quantitative method. The EVs pellet (20 µL) was mixed with 10 µL of a solution of 5 × sodium dodecyl sulfate (SDS) and 20 µL of 1 × PBS and then boiled at 100°C for 10 min. After returning to room temperature, the protein samples were centrifuged briefly and routinely Western blotted. The experiment was completed through the steps of electrophoresis, membrane transfer, blocking, antibody incubation. The primary antibodies corresponding to the target protein were CD63 (1:1000) (Abcam, USA) and Enolase (1:1000) (Cell Signaling Technology, Danvers, MA, USA). For all secondary antibody incubations, horseradish peroxidase–conjugated or goat anti–rabbit antibodies (Abcam, USA) were used at a 1:5,000 dilution. The membranes were visualized using an Gel Doc XR+ Imaging System (Bio-Rad, USA).

**Quantitative Reverse Transcription–Polymerase Chain Reaction (qRT-PCR)**

The collection of the worm samples took place on the 3rd, 55th and 80th days of in vitro culture, and were established as three experimental groups PSC, MC, and HC. The extraction of total RNA and small RNA from PSCs and MCs were the same as the preparation for high-throughput sequencing. The extraction process of total RNA from EVs requires an additional step of mixing 10 µL of nucleic acid precipitation Acryl Carrier (Solarbio, China) in isopropanol. The purity and concentration of nucleic acid samples were detected by the Nucleic Acid Protein Analyzer Nanodrop1000 (Thermo Scientific, USA). U6 and GAPDH were selected as internal reference genes. The primers were designed by Primer Premier 5.0 and synthesized by Sangon Biotech Co., Ltd. (ShangHai, China). The sequences were shown in Table 1. The qRT-PCR verifications were completed by the Mir-X miRNA First-Strand Synthesis Kit and the Mir-X miRNA qRT-PCR TB Green® Kit (Clontech, Takara, Japan) with a real-time fluorescent quantitative PCR instrument (Roche LightCycler480 II, USA). The reaction conditions were as follows: 95°C for 3 min, 1 cycles; 95°C for 5 s and 60 °C for 45 cycles. Each sample was set up with 2 replicate wells, the numbers of cycles (Ct value) at which the fluorescence signal reached the threshold were recorded, and the target genes were quantified by the 2-ΔΔCt method.
Table 1
Primer sequence.

| Gene               | Primer 5’ to 3’                      |
|--------------------|--------------------------------------|
| egr-miR-4989-3p    | RT CTCAACTGGTGTCGTTGAGCGCAATTCAGTTGAGTCTCAGAT |
|                    | F ACACTCCAGCTGGGAATATGCACCAACT       |
| egr-miR-124b-3p    | RT CTCAACTGGTGTCGTTGAGCGCAATTCACTTGAGGATTTGATTC |
|                    | F ACACTCCAGCTGGGAATATGCACCAACT       |
| egr-miR-9-3p       | RT CTCAACTGGTGTCGTTGAGCGCAATTCACTTGAGGATTTGATTC |
|                    | F ACACTCCAGCTGGGAATATGCACCAACT       |
| egl-m0694-3p       | RT CTCAACTGGTGTCGTTGAGCGCAATTCACTTGAGGATTTGATTC |
| Universal primer   | R TGGTGTCGTTGAGGTCG                   |
| U6                 | F CAGAATGCCTGCGTTGTTG                |
|                    | R TGGGATTTAAGGGCTCTGC                |
| EgrG_000892700     | F CGGCATTCTGGCTCTTACTT               |
|                    | R AAGCCCGGTTCGTGTGTGG                |
| EgrG_001029400     | F AATGCCTCACTTCGTTCTCTTTCC          |
|                    | R ACCGCCTTTGAGTGTGGGT                |
| EgrG_001081400     | F GGAATAGTTTGTCTCGGTCGTA            |
|                    | R CAATGCCGCTGGATAGGTTG               |
| EgrG_000465800     | F TCAAGAGATGACCTTCTCTACG            |
|                    | R GCTGTGCTAAAGGAAGTTGGA             |
| GAPDH              | F TGAAGATGACCTTCTCTAGTGCC           |
|                    | R TTTTCTCATCCTTACCAATCGTCT           |

(RT: specific reverse transcription primers; F: forward primers; R: reverse primers)

Result

Morphological observation of the cystic model of E. g protoscoleces in vitro

The initial PSCs presented an irregular elliptical body with a diameter of 180-220 µm. Most of the scolex were inverted, and the body contains a large number of high refractive calcium particles. The active worms showed strong refraction and slight peristalsis under the microscope, the dead worms shrunked
and became smaller, and the internal structure of which was blurred. With the prolongation of in vitro culture time, more and more scolex of worms turned out, the worms became larger and gradually vacuolated. Calcium particles, suckers, hooks and other structures disappeared, and the cuticle layer on the surface of the body wall usually appeared after 20 days of culture. There were two peaks of encystation on the 7th and 55th days after PSCs cultured in vitro. After 80 days of culture in vitro, the encystation rate of the worms reached 96.19%, the average diameter of the MCs reached 512.03±12.89 µm, and the maximum diameter reached 846.33 µm. The laminated layer of the MCs was obviously thickened, and the cysts were highly transparent (Figure 1).

**Target Prediction and Functional Annotation of the differential miRNAs between PSC and MC**

Through the analysis of the miRNAs library, 39 conservative miRNAs with differences between PSC and MC were screened. Compared with PSC, 19 miRNAs were up-regulated and 20 genes were down-regulated in MC (Table 2). Furthermore, 9439 target mRNAs were predicted (Figure 3A). In the transcriptome library, 1586 mRNAs with differences between PSC and MC were screened. Among them, 667 differential mRNAs were up-regulated and 919 differential mRNAs were down-regulated in MC (Figure 3B). The predicted 9439 target differential mRNAs were intersected with 1586 differential mRNAs in the transcriptome, and finally 1445 intersecting mRNAs were found (Figure 3C).
Table 2
Differential conservative miRNAs between PSC and MC

| id            | Expression | Expression | log2FoldChange | P-Value       |
|----------------|------------|------------|----------------|---------------|
| egr-miR-9-5p   | Up         | 4498924.605| 3.6058399      | 3.74707E-87   |
| egr-miR-4989-3p| Up         | 350179.5597| 2.3019225      | 9.18045E-48   |
| egr-miR-745-3p | Up         | 321765.4244| 1.2552085      | 1.38822E-15   |
| egr-miR-190-5p | Up         | 39455.88449| 1.311226       | 2.31018E-27   |
| egr-miR-277a-3p| Up         | 26038.41881| 2.4797341      | 4.28922E-53   |
| egr-miR-9-3p   | Up         | 11663.7998 | 2.2607011      | 9.6263E-100   |
| egr-miR-4989-5p| Up         | 1616.215122| 1.7540806      | 8.39987E-57   |
| egr-miR-31-5p  | Up         | 233.1949683| 4.18534        | 2.932E-103    |
| egr-miR-10293-3p| Up       | 167.4000474| 2.3106971      | 4.85792E-34   |
| egr-miR-31-3p  | Up         | 35.58733562| 3.9824845      | 4.07891E-18   |
| egr-miR-10256-5p| Up        | 35.20842533| 3.0961448      | 8.30042E-18   |
| egr-miR-10249-3p| Up       | 27.62381681| 2.7159161      | 1.01961E-11   |
| egr-miR-10227a-5p| Up      | 24.86525902| Inf            | 2.35965E-30   |
| egr-miR-2d-5p  | Up         | 22.70304422| 2.4468508      | 8.62082E-09   |
| egr-miR-10250-5p| Up        | 15.03858479| 1.3552199      | 0.014923507   |
| egr-miR-10229a-5p| Up       | 10.85726947| 1.5320588      | 0.010516398   |
| egr-miR-10233-3p| Up        | 9.472685265| 1.6829258      | 0.003820878   |
| egr-miR-10227e-5p| Up       | 6.21888502 | 2.950188       | 0.000306368   |
| egr-miR-10229b-3p| Up       | 2.601984138| 3.6107723      | 0.027096962   |
| egr-miR-7b-5p  | Down       | 350149.842 | -1.028968      | 2.60492E-24   |
| egr-miR-7-5p   | Down       | 253031.4958| -2.020921      | 8.16299E-90   |
| egr-bantam-3p  | Down       | 224954.3298| -1.922683      | 4.75633E-80   |
| egr-miR-125-5p | Down       | 86145.99022| -1.36707       | 1.52958E-31   |
| egr-miR-281-3p | Down       | 24869.92996| -1.304459      | 8.44703E-32   |
| egr-miR-124b-3p| Down       | 11735.77122| -1.239648      | 9.55472E-36   |
| egr-miR-3479b-5p| Down      | 1140.850684| -2.915039      | 4.34144E-73   |
| id             | Expression* | basemean     | log2FoldChange | P-Value       |
|----------------|-------------|--------------|----------------|---------------|
| egr-miR-153-3p | Down        | 757.6793838  | -1.112242      | 1.22334E-13   |
| egr-bantam-5p  | Down        | 505.6211807  | -1.247398      | 8.11431E-20   |
| egr-miR-133-5p | Down        | 290.2134118  | -2.019615      | 5.62178E-37   |
| egr-miR-1992-3p| Down        | 87.21406315  | -1.465889      | 9.46044E-09   |
| egr-miR-10234-5p| Down       | 82.26253195  | -2.631287      | 3.41441E-15   |
| egr-miR-10243-5p| Down       | 54.42111791  | -1.91383       | 1.62529E-10   |
| egr-miR-219-3p | Down        | 36.48777043  | -1.182162      | 0.000482997   |
| egr-miR-7-3p   | Down        | 25.51186714  | -1.186466      | 0.008253918   |
| egr-miR-10235-5p| Down       | 23.17492432  | -1.070468      | 0.023515598   |
| egr-miR-124a-5p| Down        | 16.18188473  | -1.415397      | 0.005324637   |
| egr-miR-281-5p | Down        | 14.4362523   | -1.185215      | 0.031312757   |
| egr-miR-10234-3p| Down      | 12.43880696  | -3.175837      | 2.34158E-05   |
| egr-miR-10235-3p| Down      | 9.930642114  | -2.842916      | 0.000179547   |

**basemean**: the normalized average expression of the overall gene of PSC and MC;

**log2FoldChange**: the log2 logarithmic value of the multiple of the gene expression difference between PSC and MC;

**Inf**: the calculated result is infinitely large or infinitely small and cannot be displayed.

*:Compared with PSC.

1430 entries were generated from 1445 candidate target mRNAs by GO enrichment analysis. The three categories of Go term from left to right are cellular component (CC), molecular function (MF) and biological process (BP). GO enrichment entries from each GO category showing the top 10 (Figure 3). 18 entries with $P<0.05$ and FDR (corrected value of P value)<0.05 were selected from the above 30 entries. Through GO enrichment analysis, it was found that the target mRNAs, which related to the transport channel activity, transmembrane transport protein activity and the molecular functions of other proteins and enzymes in the MF category, accounted for 55.56%. The target mRNAs related the CC category accounted for 27.78%, such as the occurrence of membranes. The target mRNAs which related to microtubule formation and ion transport across membranes in the BP category accounted for 16.67%.
Table 3
GO analysis of target genes ($P<0.05$)

| Category | Term                                | GO ID               |
|----------|-------------------------------------|---------------------|
| CC       | integral component of membrane     | GO:0016021;         |
|          |                                     | GO:0031224          |
| CC       | membrane                            | GO:0016020          |
| CC       | membrane part                       | GO:0044425          |
| CC       | Golgi membrane                      | GO:0000139          |
| MF       | ion channel activity                | GO:0005216          |
| MF       | channel activity                    | GO:0015267          |
| MF       | passive trans membrane transporter activity | GO:0022803      |
| MF       | substrate-specific channel activity | GO:0022838          |
| MF       | transporter activity                 | GO:0005215          |
| MF       | ion transmembrane transporter activity | GO:0015075      |
| MF       | serine-type endopeptidase inhibitor activity | GO:0004867    |
| MF       | nucleobase-containing compound kinase activity | GO:0019205  |
| MF       | receptor activity                   | GO:0004872          |
| MF       | molecular transducer activity       | GO:0060089          |
| BP       | microtubule-based process           | GO:0007017          |
| BP       | transmembrane transport             | GO:0055085          |
| BP       | ion transport                        | GO:0006811          |

The gene function Clusters of candidate target mRNAs were further analyzed by KEGG database system. 1445 differential mRNAs were clustered into 94 metabolic pathways by KEGG enrichment analysis, which included cellular process (CP), environmental information processing (EP) and metabolism (M). The first 30 pathways with the lowest $P$ in KEGG classification were selected and displayed in the form of bar graph (Figure 4). 25 pathways of the first 30 pathways of target mRNAs enrichment were related to metabolism. There were amino acid or nucleotide metabolic pathways, including arginine metabolic pathway (egl00220), arginine and proline metabolic pathway (egl00330), purine metabolic pathway (egl00230), pyrimidine metabolic pathway (egl00240), alanine, aspartic acid and glutamate metabolic pathway (egl00250). There were also glycan biosynthesis pathway (egl00512,egl00515), nitrogen metabolism pathway (egl00910), carbohydrate metabolism pathway (egl00052,egl00040), fat
metabolism pathway (egl00071, egl00100, egl00061), vitamin B6 metabolism pathway (egl00061). The Notch signaling pathway was among the only two EP-related pathways, and it was also listed at the 17th place of the 18 pathways with the most significant enrichment ($P < 0.05$). Another EP-related pathway was the Neuroactive ligand-receptor interaction pathway. Compared with the Notch pathway ($P = 0.040498$), this pathway was more enriched ($P = 0.002529$) and worthy of attention.

**Prediction and screening of differential new miRNAs during the encystation of E. g PSCs.**

After matching the following 3 conditions: the precursor secondary structure was a classic stem-loop structure (egl-m0694 as an example, Figure 5), minimum folding free energy (MFE) $<-20$ kcal/mol, minimum folding freedom Energy Index (MFEI) $>0.85$, 50 new miRNAs were screened. Through the differential analysis of the expression of these new miRNAs between PSC and MC, six differential new miRNAs were screened out (Table 4). Compared with PSC, 5 new miRNAs are down-regulated in MC and 1 is up-regulated. Among them, egl-m0694-3p has the highest expression level among the 50 new miRNAs and the down-regulated differential new miRNAs. Its role in the encystation deserved further study.

**Table 4 The differential new miRNA**

| id          | Expression* | baseMean | log2FoldChange | $P$-value  |
|-------------|-------------|----------|----------------|------------|
| egl-m0694-3p| DOWN        | 1044715  | -1.272161      | 0.0006846  |
| egl-m0755-3p| DOWN        | 214.6396 | -1.843311      | 0.0003181  |
| egl-m0024-5p| UP          | 20.719225| 2.0352186      | 0.0017998  |
| egl-m0517-3p| DOWN        | 18.840022| -1.318678      | 0.045803   |
| egl-m0712-5p| DOWN        | 5.2830587| -3.669683      | 0.0114489  |
| egl-m0219-3p| DOWN        | 2.188075 | -Inf           | 0.0492107  |

**base-mean**: the normalized average expression of the overall gene of PSC and MC;

**log2FoldChange**: the log2 logarithmic value of the multiple of the gene expression difference between PSC and MC;

**Inf**: the calculated result is infinitely large or infinitely small and cannot be displayed.

*: Compared with PSC.

**Notch signaling pathway related miRNA-mRNA subnetwork and candidates for miRNAs**

Through the KEGG enrichment analysis, 4 target mRNAs predicted by the conservative differential miRNAs between PSC and MC were enriched in the Notch signaling pathway. The target mRNAs were annotated through the Swissprot database. Corresponding to the Notch regulatory pathway, these mRNAs
are all important regulatory genes of the Notch signaling pathway (Table 5). 37 conservative differential miRNAs related to these 4 target mRNAs were further found. Compared with PSC, 18 were up-regulated in MC and 19 were down-regulated. Finally, with the rich differential expression and the number of connections with the target gene (degree) $\geq 3$ as the screening conditions, a miRNA-mRNA subnetwork of 4 conservative differential miRNAs corresponding to 4 target mRNAs was formed (Figure 7). Been predicted to be related to EgrG_001029400, the new miRNA egl-m0694-3p with the highest differential expression also became one of the candidate miRNAs.

Table 5 Annotation of Target mRNAs Related to Notch Signaling Pathway

| id                | Expression* | Protein annotation                          | Notch Pathway annotation |
|-------------------|-------------|---------------------------------------------|--------------------------|
| EgrG_000892700    | Down        | Adisintegrin and metalloproteinase17, ADAM17| TACE                     |
| EgrG_001029400    | Up          | Suppressor of hairless protein, SUH        | CSL                      |
| EgrG_001081400    | Up          | C-terminal Binding Protein, CtBP            | CtBP                     |
| EgrG_000465800    | Up          | Delta-like ligand 4, DLL4                  | Delta                    |

*:Compared with PSC.

Identification of EVs from PSCs

Transmission electron microscopy (TEM) identified vesicles with a cup shape morphology, which was characteristic of EVs (Figure 8A). Particle size measurements obtained using Nanoparticle Tracking Analysis (NTA) showed that the majority of purified vesicles derived from PSCs were between 40–150 nm in diameter (Figure 8B). As marker proteins of EVs, both CD63 and enolase were verified by Western blotting (Figure 8C).

qRT-PCR validation of candidate genes

The trends of differential mRNAs expression validated by qRT-PCR were consistent with the high-throughput sequencing results. Compared with PSC group, EgrG_001081400, EgrG_000465800 and EgrG_001029400, the expressions of these three target genes in Mc group were up-regulated, while the expression of EgrG_000892700 was down-regulated ($P<0.01$). Except the expression of EgrG_001081400 was significantly higher in HC group than that in MC group, the expressions of the other three mRNAs were below the lower limit of PCR detection (Figure 9A). Except that EgrG_001029400 was not expressed in the EVs of PSC, the three up-regulated expression mRNAs were all verified in the three-phase EVs samples, and the change trends of the expression were the same as the change trends of the worm (Figure 9B).
The results of the miRNA-mRNA subnetwork analysis were predicted and further verified by qRT-PCR. Among them, egr-miR-4989-3p, egr-miR-9-3p and egr-miR-277a-3p might have negative regulatory effects on EgrG_000892700; egr-miR-124b-3p and egl-m0694 -3p might have a negative regulatory effect on EgrG_001029400; egr-miR-124b-3p might have a negative regulatory effect on EgrG_001081400. Except for the slight decrease of egr-miR-9-3p in HC group, the expression levels of egr-miR-4989-3p, egr-miR-9-3p and egr-miR-277a-3p showed an upward trend in the worms during the encystation process, while egr-miR-124b-3p and egl-m0694 -3p showed a downward trend. The above qRT-PCR verification results were consistent with the high-throughput sequencing analysis results(Figer 9C).

The expressions of egr-miR-4989-3p, egr-miR-277a-3p and egl-m0694 -3p in EVs from MC group were higher than that from HC group. Among them, The expressions of egr-miR-4989-3p and egr-miR-277a-3p in EVs from MC group were both close to 5 times that of EVs from HC group, while no statistically significant difference of the expressions of the above two miRNAs in EVs between PSC group and HC group. The expression of egr-miR-9-3p had no statistically significant difference in EVs from PSC group and EVs from MC group, and decreased slightly in EVs from HC group (Figer 9D).

Discussion

Although surgery and imidazole drugs are the main methods for the treatment of CE, blocking the encystation and development of protoscoleces in the host is the key to avoiding secondary infections and postoperative recurrence. Therefore, the highly plastic bi-directional development of protoscoleces is the focus of the study of the developmental mechanism of *E.g*[11]. Protoscolex is an important stage of development in the complex life cycle of *E.g*, which can develop into adults in the gastrointestinal tract of the terminal host and echinococcosis cyst in the liver and lung of the intermediate host. Even if cultured in MEM basal medium without additional components such as serum for 15 days, the in vitro encystation rate of protoscoleces might reach to 35.65%[12], indicating that developmental regulation is still the main mechanism of parasite morphological changes. Microcysts of *Echinococcus granulosus* cultured in vitro for more than 60 days could significantly improve the modeling rate of intraperitoneal inoculation infection model[13–14]. Compared with PSCs, MCs were more active in increasing IL-6 levels to promote the chronic infection process of hydatid and down-regulating IFN-γ levels to promote the survival of worms[15], suggesting that MCs might play a key role in secondary hydatid infections. Wnt or Notch, who has the main regulatory role in the process of encystation of *E. g* PSCs? Weather extracellular vesicles participate in the regulation of *E. g* encystation by targeting non-coding RNAs related to developmental pathways? The above-mentioned scientific hypotheses are urgently to be verified.

Non-coding RNA plays an important role in the physiological and pathological processes of eukaryotes[16]. The seed sequence of mature miRNAs is 2 to 7 nucleotides long and has been considered to be the most critical region for recognizing target mRNAs, IncRNAs and CircRNAs through complementary base pairs. Therefore, miRNA is a key regulator of worm-host interaction[17].
transcriptional regulation of miRNAs is one of the key mechanisms for controlling animal and plant development. It participates in the post-transcriptional regulation of almost all cellular signal pathways in animals and plants[18] and is widely expressed in *E. multilocularis*, and *Echinococcus canadensis*[19-20]. Both Bai, Y[21] and Cucher, M[5] reported that MiR-2, miR-71, and miR-125 have the highest expression levels among the 76 known miRNAs of *E. granulosus* sensu stricto. In addition, miRNAs were found to exhibit tissue- and phase-specific expression. MiR-277, let-7, miR-71, miR-10, miR-2, and miR-9 are specifically expressed in the cysts walls of secondary hydatid cyst and protoscoleces of G1 and G7 genotype, whereas miR-125 is only detected in protoscoleces and pre-microcysts. Additionally, three miRNAs (let-7, miR-71, and miR-2) are expressed at high levels in protoscoleces of metacestodes (cyst walls), which suggests that their expression is developmentally regulated[22]. Gene Ontology (GO) enrichment analysis revealed that the differentially expressed miRNAs in *E. granulosus* and their potential targets may participate in nutrient metabolism and bi-directional development of the nervous system[23]. By analyzing the mechanism of miRNAs regulating cystic development in *E. granulosus*, it is possible to find the target to control the encystation of *Echinococcus* and trigger CE[24].

High-throughput sequencing technology has enabled the research on non-coding RNAs of *Echinococcus* to be enthusiastically carried out, and the mapping of the miRNA-mRNA subnetwork makes the prediction of the function of the gene in the corresponding regulatory pathway more accurate[25]. The establishment of the bi-directional development model of PSC in vitro to adult and MC and the difference of observation period often leaded different research conclusions. Our study found there were two peaks of encystation on the 7th and 55th days after PSCs cultured in vitro. Bai, Y[6] employed RNA and small RNAs sequencing to characterize the gene and miRNA expression at 0–24 h and 7–14 days in the bi-directional development of PSCs. A total of 963 genes and 31 miRNAs were differentially expressed in the early development of PSCs to adult worms whereas 972 genes and 27 miRNAs were differentially expressed in the early development of PSCs to MCs. Pairwise comparison between the two developmental patterns showed that 172 genes and 15 miRNAs were differentially expressed at three time-points. Most of these genes were temporally changed at 24 h or 7 days. GO enrichment analysis revealed that the differentially expressed genes in early adult worm development were associated with nervous system development and carbohydrate metabolic process; whereas, the differentially expressed genes in early cystic development were associated with transmembrane transporter activity and nucleoside triphosphatase activity. In our study, the GO enrichment analysis showed 55.56% of the target mRNAs related to the transport channel activity, transmembrane transport protein activity and the molecular functions of other proteins and enzymes in the MF category. Part of these conclusions are consistent with previous reports.

Fan J[26] also established an in vitro encystation model of up to 80 days in the study, and further screened out 32,401 transcripts and 14,903 cDNAs revealed numbers of new genes and transcripts, stage-specific genes, and differently expressed genes in the encystation process with an in vitro culture cycle separated by 20 days. The GO classification of function-enriched 1,991 DEGs showed that 1,094, 148, and 263 DEGs were assigned to the cellular component (CC), biological process (BP), and molecular function (MF) categories, respectively, of which 7090, and 122 genes were significantly differentially
expressed. More than half of the DEGs were assigned to CC category, which included integral components of membrane, intrinsic components of membrane, and membrane part.

After analyzed by KEGG database system, 1445 differential mRNAs were clustered into 94 metabolic pathways, which included CP, EP and metabolism (M) categories. Among of them, 25 of the top 30 pathways with the most significant enrichment associated with metabolism. This result is consistent with Zheng's report[27], which showed *E. granulosus* had 500–550 KEGG ontology terms associated with metabolism, and had complete pathways for glycolysis, the tricarboxylic acid (TCA) cycle and the pentose phosphate pathway.

*E.granulosus* possesses several complete signaling pathways, including for mitogen-activated protein kinase (MAPK), ErbB, Wnt, Notch, Hedgehog, TGF-β, Jak-Stat and insulin signaling[28]. On the proteomic analysis of PSC[29], Among the 1,197 proteins expressed by protocephala, 632 proteins are involved in 276 regulatory pathways, such as the Wnt, Notch, Hedgehog, NF-κB, cAMP and bile acid signaling pathways closely related to the development of *E. granulosus*. The β-catenin was mainly distributed in the hooks and dispersed cells of the PSCs. Meanwhile, the relative transcription of β-catenin was gradually decreased during 0 to 10 days in vitro culture. The mRNA expression of wnt2 in adult *E. granulosus* was higher than that in PSC, and the mRNA expression of wnt4, wnt5, wnt11A and wnt11B in PSC was higher than that in the adult worms. The six wnt gene family members were all distributed in the forward region of PSCs[30]. The Wnt signaling pathway may play an important role in the formation of *E.g* body axis, and at the same time may play an important regulatory role in the development of the body’s germ cells.

In this study, the Notch pathway instead of the Wnt pathway, through KEGG analysis, was one of the 18 pathways with the most significant enrichment, and also one of the only two EP-related pathways in the top 30 pathways with the most significant enrichment. Notch signaling pathway is found in organisms as diverse as worms and humans. The notch gene plays a critical role in tissue development, regulation of cell proliferation, and differentiation and cell fate in all metazoans[31–32]. Dezaki[33] found The notch gene was expressed at all developmental stages of *E. granulosus*, however, the fold difference was significantly increased at the cultivated in vitro and the germinal layer of hydatid cyst in comparison with other stages. Although the two pathways are related to each other[34], the Wnt signaling pathway may be more involved in the development of PSCs to adults, while the Notch signaling pathway may be more active during the encystation of *E.g* PSCs.

A miRNA-mRNA subnetwork of 5 differential miRNAs corresponding to 4 target mRNAs was formed, which enriched in the Notch signaling pathway. The 4 target mRNAs are respectively annotated as TACE, CSL, CtBP, and Delta, which are all important components of the Notch signaling pathway. Although the other three mRNAs tended to be up-regulated during encystation process, EgrG_000892700, annotated as ADAM17, was down-regulated during encystation process. Notch signaling is a highly conserved pathway regulating cell fate decisions in embryonic development and adult tissue homeostasis. After binding of the ligands (Jagged1, 2 and Dll1, 3, 4 in mammals), Notch receptors (Notch 1-4) undergo two proteolytic
cleavages catalyzed by ADAM-family metalloproteases and γ-secretase, resulting in the release of the intracellular domain of Notch receptors (ICNs) from the cell membrane. ICNs then enter the nucleus where they bind to the transcription factor CSL (CBF1, Su(H) and LAG-1) and activate transcription of Notch target genes[7]. ADAM17 can mediate S2 cleavage under special, nonphysiological circumstances. It cleaves T-ALL-associated mutant forms of Notch that are ligand independent in tissue culture cells[35]. ADAM10 is necessary for Notch processing when Notch is activated by a ligand, while ADAM17 is the major protease for processing Notch that is activated independently of ligand in both flies and mammals[36]. If the expression of ADAM17 is down-regulated, the level of NICD in the nucleus and the accumulation of NICD will be significantly reduced, the transcription and cell proliferation regulated by the Notch signaling pathway will be inhibited, which is mutually corroborated with the physiological characteristics that PSC does not proliferate but encyst in vitro. Constitutive activation of Notch by expressing NICD in the developing chick heart promoted conduction cell differentiation and inhibited cardiomyocyte differentiation, while inactivating the Notch pathway by expression of a dominant negative version of Su(H) increased myocardial lineage markers at the expense of conduction cell markers[37]. Activation and inhibition of the Notch signaling pathway may guide the differentiation outcomes of different cells, and its role in the regulation of E.g encystation needs to be further explored.

Based on the characteristics of miRNA negative regulation of mRNA, egr-miR-4989-3p, egr-miR-9-3p and egr-miR-277a-3p have potential negative regulatory effects on EgrG_000892700, which were verified by PCR. These three miRNAs may be involved in the inhibition of ADAM17 expression, thereby affecting the downstream transcriptional regulation of the Notch signaling pathway. Although the role is not clear, the new miRNA egl-m0694-3p was predicted to only have potential negative regulatory effect on EgrG_001029400 in the subnetwork, and whose expression was significantly down-regulated during the process of encystation.

This study further focused on the transport of five miRNAs related to the Notch signaling pathway in the extracellular vesicles (EVs) of PSC during the process of encystation. The research on EVs of worms lags behind that of protozoa. As the first report on extracellular vesicles of parasitic worms in 2012, Marcilla[38] found that there are extracellular vesicles in the envelope and excretory secretions of *Fasciola hepatica*. Subsequently, reports of EVs of various trematodes and filaria[39], whipworm[40], roundworm[41] and other nematodes appeared one after another. Previous studies[42–43] showed that both adults and eggs of *Schistosoma japonicum* can secrete EVs. 403 proteins, 15 known *S. japonicum* miRNAs and 19 new miRNAs were detected from the EVs of *Schistosoma japonicum*. The EVs of *Schistosoma japonicum* can be internalized by mammalian cells, and the proteins and miRNAs contained in them can be transferred to Recipient cells down-regulate the expression of corresponding target genes. Wang[44] found that the EVs of *Schistosoma japonicum* contained a special excretion secretory protein, which can mediate the immune activity of macrophages M1 type and increase the secretion of pro-inflammatory cytokines TNF-α and IL-12. The reports on EVs of cestodes are currently limited to *Taenia asiatica*[45], *Taenia saginata* and *Echinococcus*[19](Ancarola et al., 2017), and mainly focusing on interactions between EVs of cyst fluid and immune cells such as macrophages and dendritic cells[46–47]. Yang J[48] found one type of 110 K EVs from hydatid fluid (HF) of *E. granulosus*, which loaded
25 miRNAs, were internalized by sheep peripheral blood mononuclear cells (PBMCs) in a time-dependent manner and thus induced interleukin (IL)-10, tumor necrosis factor-α (TNF-α), and IRF5 were significantly upregulated and IL-1β, IL-17, and CD14 were significantly downregulated. Exosome-related miRNAs contributes to the interaction between host and parasites in worms[49] and affects host immune response[50]. However, there were few reports on the regulation mechanism of EVs-related miRNAs on the development of parasite.

118 miRNAs and 2,361 lncRNAs of EVs in PSC of E.g were identified by Zhang[10]. The types and numbers of which were more than EVs in cyst fluid. Among them, egr-miR-4989-3p has the highest content in EVs both from PSC and cyst fluid. Zheng[51](Zheng et al., 2016) confirmed that emu-miR-4989-3p has been encapsulated in EVs produced by Echinococcus multilocularis, which has a significant impact on the production of nitric oxide (NO) in macrophages and the expressions of several key components involved in the LPS/TLR4 signaling pathway, suggesting that miR-4989-3p has an immunomodulatory effect in the process of Echinococcus infection.

Consistent with Zhang's report[10], the expressions of three miRNAs, egr-bantam-3p, egr-miR-4989-3p and egr-miR-277a-3p were in EVs of PSC were verified by qRT-PCR. egr-bantam-3p down-regulated during the encystation process (not written in this article), while egr-miR-4989-3p and egr-miR-277a-3p up-regulated in EVs during the encystation process, and have a potential negative regulatory effect on the EgrG_000892700 enriched in the Notch signaling pathway. During the study, we found that the collection of EVs became more difficult as the thick of the laminated layer of the MCs in the late stage of encystation, but the loaded mRNAs and miRNAs were rich in content. After 80 days of culture in vitro, the expressions of the four mRNAs enriched in the Notch signaling pathway were all lower than the detection limit except EgrG_001081400 in MCs, but all could be detected in the EVs except EgrG_000892700. In the middle stage of encystation, the expressions of egr-miR-4989-3p and egr-miR-277a-3p in EVs of MCs were nearly 5 times that of PSCs. EVs may negatively regulate the expression of EgrG_000892700 in worms through continuous targeting of egr-miR-4989-3p and egr-miR-277a-3p, inhibit the transcription of the Notch signaling pathway, and may participate in the regulation of encystation of E. g.

### Conclusion

This study has screened out the miRNA-mRNA subnetwork that may be involved in regulating the Notch signaling pathway during the PSC encystation process through bioinformatics analysis. Contained a new miRNA egl-m0694-3p, A miRNA-mRNA subnetwork of 5 differential miRNAs corresponding to 4 target mRNAs enriched in the Notch signaling pathway was formed. The study futher verified the changes of expressions of the above genes in the worms and EVs. EVs may negatively regulate the expression of EgrG_000892700 in worms through continuous targeting of egr-miR-4989-3p and egr-miR-277a-3p, inhibit the transcription of the Notch signaling pathway, and may participate in the regulation of encystation of E.g. This study provides a preliminary basis for EVs to regulate the encystation of E. g PSCs by targeting and transporting Notch signaling pathway-related miRNAs. The effect of EVs on the encystation process after being internalized by PSCs need to be further observed, and the regulation of the targeted delivery of
corresponding miRNAs by EVs on the encystation of worms still needs to be verified by small RNA interference technology.

**Declarations**

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**Availability of data and materials**

We have raw data and sequencing results, but not uploaded to public databases.

**Author's Contribution**

YS conceived and designed the study. JG, XZ, LL, GL, QH and Xiaofan Zhang performed the experiments and data analysis. YS contributed reagents and materials. JG and XZ wrote the manuscript. YS revised the manuscript. All authors read and approved the final version of the manuscript.

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

All authors read and approved the consent for publication.

**Competing interests**

All authors have no Competing interests.

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Figures
Figure 1

An in vitro encystation model of PSCs of *E. g.*

(A-F) Morphological changes of PSCs after 3 days, 7 days, 20 days, 40 days, 60 days and 80 days of culture in vitro. Scale bar: 100 μm. (G) Average diameter of PSCs. (H) The encystation rate of PSCs.
Figure 2

Analysis and intersection of differential miRNAs and differential mRNAs (PSC vs MC)

Volcan of differential miRNAs; (B) Volcan of differential mRNAs; (C) Venn diagram based on the intersection between the target mRNAs predicted by differential miRNAs and the differential mRNAs.
Figure 3

GO analysis of target genes (Top 10 of terms)

CC: cellular component; MF: molecular function; BP: biological process.
Figure 4

KEGG analysis of target genes
Figure 5

Secondary structure of new miRNA egl-m0694 precursor

Figure 6

Notch signaling pathway

Red box: genes up-regulated in MC compared to PSC; Green box: genes down-regulated in MC compared to PSC;
Figure 7

The miRNA-mRNA subnetwork related to Notch signaling pathway

Circle: target mRNA; diamond: miRNA; red: up-regulation of expression; green: down-regulation of expression; the larger the graph area, the more genes associated with this gene.
Identifications of EVs from PSCs

Images of the rounded or cup-shaped vesicles obtained using negative staining under TEM. Scale bar: 100 nm; (B) The diameter distribution analysis of the purified EVs assessed using nanoparticle tracking analysis. (C) EVs markers CD63 and enolase were measured using Western blotting analysis.
Figure 9

Relative quantification of the target mRNAs and candidate miRNAs of Notch signal pathway

(A) Relative quantification of the target mRNAs in the worms; (B) Relative quantification of the target mRNAs in the EVs; (C) Relative quantification of the candidate miRNAs in the worms; (D) Relative quantification of the candidate miRNAs in the EVs.
PSC: protoscoleces; MC: microcyst after 55 days cultured in vitro; HC: microcyst after 80 days cultured in vitro; —: Differences between adjacent groups; ——: difference between the first group and the last group.

**: \(P<0.01\); *: \(P<0.05\); ns: \(P>0.05\)

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