Cloning and characterization of *Echinococcus granulosus* ecdysteroid hormone nuclear receptor HR3–like gene

Mei Yang[a,c], Jun Li[b], Jun Wu[d], Hui Wang[b], Baoping Guo[b], ChuanchuanWu[b], Xi Shou[b], Ning Yang[b], Zhuangzhi Zhang[e], Donald P. McManus[f], Fuchun Zhang[a,*], Wenbao Zhang[b,*]

[a] Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, College of Life Science and Technology, Xinjiang University, Urumqi 830046, PR China

[b] State Key Laboratory Incubation Base of Xinjiang Major Diseases Research ,the First Affiliated Hospital of Xinjiang Medical University, Urumqi 830011, PR China

[c] Basic Medical College of Xinjiang Medical University ,Urumqi 830011, PR China

[d] Public Health College of Xinjiang Medical University, Urumqi 830011, PR China

[e] Molecular Parasitology Laboratory, QIMR Berghofer, Herston, QLD, 4006, Australia

[f] Veterinary Research Institute, Xinjiang Academy of Animal Sciences, Urumqi, 830000, PR China

* Corresponding author. Address: Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, College of Life Science and Technology, Xinjiang University, 14 Shengli Road, Urumqi 830046, PR China. Fax: +86 0991 8583259.

E-mail address: zfcxju@xju.edu.cn; wenbaozhang2013@163.com

Abstract

Cystic echinococcosis (hydatid disease) is an important parasitic zoonosis caused by the dog tapeworm *Echinococcus granulosus*. Little is known about adult worm development in molecular level. Transcription analysis showed that *E. granulosus* hormone receptor 3-like (*EgHR*3) was expressed in protoscoleces and adult worms indicating the role of early adult development. In the study, we cloned and characterized *EgHR*3 showing that its cDNA contains an open reading frame (ORF) of 1890 bp encoding a 629 amino acid protein, which has a DNA-binding domain(DBD) and a ligand-binding domain(LBD). Immunolocalization revealed the protein was localized in the parenchyma of protoscoleces (PSC) and adult worms. Real-time PCR analysis showed that *EgHR3* was expressed significantly higher in adults than other stages($P<0.01$), especially in the early adult worm development induced by bile acids. *EgHR3* siRNA silenced 69-78% of the level of transcription in PSC, which resulted in killing 43.6-60.9% of PSC after 10 days of cultivation *in vitro*. *EgHR3* may play an essential role in early adult worm development and in maintaining adult biological processes and may represent a novel drug or vaccine target against echinococcosis.

Keywords: *Echinococcus granulosus*; hormone receptor 3; cloning; siRNA

1. Introduction

Cystic echinococcosis (CE) is characterized by the presence of hydatid cyst in the liver, lung or other organs of humans and herbivores. The cyst is the larval stage of

the dog tapeworm *Echinococcus granulosus sensu lato* (s.*l.*)(*E. granulosus*). CE is a near-cosmopolitan distribution [31] and a medical important disease and causes economic losses in the endemic communities [8, 27, 32]. Globally, 3 million people have detectable hydatid cysts [26, 40]. CE is highly endemic in western China with prevalence ranging from 2-12% [62].

The life cycle of *E. granulosus* is complex, which involves two mammals, including an intermediate host, usually domestic or wild ungulates (humans are accidental hosts), and a canine definitive host (dogs and wolves). The canine animals are infected by ingestion of animal offal harboring hydatid cysts containing protoscoleces (PSC). After ingestion, PSC evaginate, attach to the canine intestinal mucosa, and develop into adult worms [32]. One remarkable feature of the PSC is its ability to develop into either an adult worm (i.e., sexual development) in the dog tract, or a hydatid cyst (i.e., asexual development) in the intermediate (or human) host. Smyth showed that dog bile plays a crucial role in the sexual differentiation and adult development [38, 49, 50]. However, the molecular mechanisms underpinning these processes remain unknown.

Our previeous transcription analysis showed that *E. granulosus* hormone receptor 3-like (*EgHR*3) was expressed in protoscoleces and adult worms indicating the role of early adult development. Hormone receptor 3 (HR3), a nuclear receptor (NR) superfamily 1F member, is induced after puparium formation, represses early gene expression and is a direct activator of the prepupal regulator FTZ-F1(HR39)[18, 19]. HR3 is a major component of ecdysteroid signaling pathway and mediates certain

development-specific responses to ecdysteroid pulses [12].

Ecdysteroids (steroidal molting hormones of insects) have been detected and characterized in several non-arthropod phyla animals including platyhelminths, nemathelminths and coelenterates[13], such as in blood fluke worm *Schistosoma mansoni*[41, 55] and tapeworms *Moniezia expansa*[34] and *E. granulosus* [36]. Ecdysteroids play function by binding to their receptors and regulate transcriptional activity of nuclear receptors including hormone receptor (HR)3 in insect. It was proposed that these hormones play roles in strobilation, detachment of mature proglottids, vitellogenesis and embryogenesis of the tapeworms [34][36].

In this study, we cloned and characterized *E. granulosus HR3*-like (*EgHR3*) and showed that *EgHR3* was highly expressed in adult worms including early, immature adult worms induced by bile acids. Silencing *EgHR3* gene significantly impacted on the survival of PSC, indicating that EgHR3 may play an important role in the differentiation of PSC into adult worms.

2. Methods

2.1. Ethics Statement

Dogs were used for raising adult worms of *E. granulosus sensu lato (s.l)* (*E. granulosus*) using the same methods previously described[32, 61]. BALB/c mice were used for preparing anti-serum. The “Guidelines for the Care of Laboratory Animals” by the Ministry of Science and Technology of the People’s Republic of China (2006) were rigidly followed in the use of these animals. The dogs were maintained un-caged

in a special facility at the Veterinary Research Institute of Xinjiang Academy of Animal Science. They were provided with water and standard chow pellets. The strict protocols for the use of dogs and mice were approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (approval number IACUC-20120625003).

2.2. Sample preparation

1. Fresh *E. granulosus* protoscoleces (PSC) were directly aspirated from hydatid cysts present in the livers of sheep from a slaughterhousein Changji, Xinjiang, China. The PSC were washed 6 times with PBS before use.

2. Freshly obtained *E. granulosus* adult worms were collected from humanely euthanized dogs 35 days post-infection with PSC as described [61]. The worms were washed 10 times with PBS and directly stored at -80^{o}C or soaked in RNAlater (Sigma, Saint Louis, MO) and stored at -80^{o}C until use.

2.3. Total RNA extraction and cDNA synthesis

PSC (100 µl) were used for extracting total RNA using 1 mL TRIzol Reagent (Invitrogen, Carlsbad, CA) and total RNA was extracted according to the manufacturer's instructions and details based on our our previous study [Li, J., W. B. Zhang, et al. (2004). "Recombinant antigens for immunodiagnosis of cystic echinococcosis." Biol Proced Online **6**: 67-77]. After being treated with RNase-free DNase I (Thermo Fisher Scientific, Waltham, MA,) for 30 min at 37^{o}C, to remove possible genomic DNA contamination, the RNA was assessed for purity by electrophoresis on a 1.5% (w/v) agarose gel. First strand cDNA was synthesized using 100 ng of total RNA with a RevertAid reverse transcriptase kit (Thermo Fisher

Scientific, MA) using Oligo (dT)18 (0.5 mg/mL) as the anchor primer. The reaction mixture was incubated at 42^{o}C for 1 h and then at 70^{o}C for 5 min to terminate the reaction. The final cDNA product was diluted 5-fold with nuclease-free water prior to its use in PCR analysis [10].

2.4. EgHR3 cloning and expression

In previous genome studies [57, 64], two genes (GenBank accession no.EUB60779.1 and CDS18863.1) encoding DBD and LBD sequence of HR3-like protein (EgHR3) respectively in *E. granulosus* were predicted. The gene alignment analysis showed these two predicted fragments may come from gene *EgHR*3. To amplify the full-length of EgHR3, a pair of primers were designed were designed (EgHR3-F: 5′ ATGCTATGTCTCGTATGCGGAGACA3′ and EgHR3-R: 5′ CTAGACAAGAGAAAAGGTTTCGCTATACA3′) and the full-length of cDNA was amplified by PCR. To expression DNA binding domain （DBD）of the protein, a couple of primers containing restriction enzyme sites of *Bam*H I and *Not* I (italicised letters) were designed to clone into pET 30 vector: *EgHR3*-DBD-F: 5′cgc*GGATCC* ATGCTATGTCTCGTATGCGGAGACACT3′ and *EgHR3*-DBD-R: 5′ataagaat*GCGGCCGC*CTATTTAGATATCTTTTTAGCGCTACATCTGCC3′. The amplified PCR products were digested with restriction enzymes and cloned into an expression vector pET-30a(+)(Invitrogen) with a 6 histidine (His) fusion tag. The inserted sequence was verified by DNA sequencing. The resulting plasmid was transformed into *E. coli* BL21 (DE3) cells. The expression of *EgHR3-DBD* was induced with 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 22°C for 20 h.

The recombinant fusion protein (rEgHR3-DBD) was purified using a His tagged affinity column (Invitrogen). Polyclonal antiserum against rEgHR3-DBD was generated using BALB/c mice. Each mouse was subcutaneously immunized with 25 μg of the purified recombinant protein emulsified with complete Freund's adjuvant as primary immunization. This was followed by 2 subcutaneous injections of rEgHR3-DBD and 2 intraperitoneal injections as boosts with the same dose of rEgHR3-DBD emulsified with incomplete Freund's adjuvant with a two week interval between injections. Blood was collected for serum preparation one week after the final immunization. The serotiter of the antiserum against the recombinant protein was determined by enzyme-linked immunosorbent assay (ELISA).

For Western blotting, the recombinant protein was separated by SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore 0.45μm) at 80 V for 1.5 h. The PVDF membrane was blocked with 5% (w/w) skim milk in PBS for 2 h at room temperature, washed 5 times with PBS containing 0.1% (v/v) Tween 20 (PBST, pH 7.4), and then incubated with a His-Tag monoclonal antibody (mAb-His) (Sigma-Aldrich, USA, 1:2,000 dilution) or anti-rEgHR3-DBD antiserum (1:400 dilution) overnight at 4°C. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma, MA, 1:2,000 dilutions) for 1 h at 37°C after 5 washes with PBST. Color was developed with 4-chloro-1-naphthol substrate solution at room temperature.

2.5. Immunoblotting for probing E. granulosus native proteins

For extracting native proteins, parasite tissues (PSC, adult worms and cyst germinal

layer) of *E. granulosus* were collected. *E. granulosus* cyst germinal layer was prepared according to our previous study [64].The parasite tissue was suspended in lysis buffer (Beyotime, Shanghai, China) containing 1 mM PMSF (Sigma), homogenized using a homogenizer for 5 min and then sonicated on ice until the suspension became clear. The homogenate was centrifuged at 12,000 rpm at 4°C for 60 min and the supernatant containing soluble proteins was retained. To extract insoluble proteins, the tissue pellets were re-suspended with lysis buffer containing 1% (w/v) SDS, and heated at 60°C for 20 min .

The native *E. granulosus* proteins were separated through 12% SDS-PAGE gels and Western blot analysis was used to identify the protein in the parasite.

2.6. Mass spectrometry of rEgHR3-DBD

To determine whether rEgHR3-DBD was the correct target protein, the recombinant polypeptide was electrophoresed on a 12% SDS-PAGE gel and then stained with Commassie Blue. The target band putatively containing rEgHR3-DBD was cut and gel strips were sent to The Beijing Genomics Institute (BGI, Shenzhen) for proteomic sequencing analysis.

2.7. Immunofluorescence analysis of EgHR3 in adults and PSC of E. granulosus

PSC and adult worms of *E. granulosus* were separately fixed in 4% (v/v) paraformaldehyde buffered in PBS. The fixed parasites were dehydrated in ethanol solution, embedded in paraffin and sectioned. The sections on slides were de-paraffinized in xylene and then ethanol. Affinity-purified mouse anti-rEgHR3-DBD serum (1:400) was used as the first antibody to probe native

proteins in the parasite tissues. After 5 washes with PBS, conjugated goat anti-mouse fluorescent antibody-CF^{TM}568 (diluted 1:800 with 0.1% BSA in PBS, Sigma) was loaded onto the slides which were incubated for 3 hours at 37°C. Pre-immune serum from the mice immunized with rEgHR3-DBD served as a negative control. After rinsing four times with PBST (5 min each) the slide sections were stained with the fluorescent nuclear stain DAPI for 10 min, and then rinsed a further four times with PBST. The slides were then imaged using a Leica TCS SP8 Confocal laser scanning microscope.

2.8. Bioinformatics analysis of EgHR3-like

The physicochemical properties of EgHR3 were predicted by the ProtParam tool (http://www.expasy.ch/tools/protparam.html). Functional domains and motif sites were analyzed by Motifscan (http://myhits.isb-sib.ch/cgi-bin/motif_scan). Bioedit was used to analyze the homology of the DBD and LBD of the target sequence. Data for all orthologous proteins were collected using Blast explorer according to the highest scores among the top 100 hits. A phylogenetic tree of the DBD of the protein family was constructed using the MEGA6.0 neighbor-joining algorithm and the Maximum Likelihood (ML) method (1000 replicates) [23]. All positions containing gaps and missing data were eliminated. Evolutionary distances were computed by the Poisson correction method.

2.9. Quantitative real-time PCR analysis of EgHR3-like expression

E. granulosus PSC were washed 6 times with 1×PBS (PSC sample), treated with pepsin and trypsin and cultured for 3 hours (PSC-3h) to 2 weeks (PSC-2w) in Smyth's

published culture system [49, 50] with or without sodium taurocholate in parallel.

Total RNA was extracted from PSC, cultured cyst germinal layer (CM) [64], and 35 day adult worms [61] of *E. granulosus* using TRIZOL (Invitrogen) according to the manufacturer's instructions. A RevertAid reverse transcriptase kit (Thermo Fisher Scientific) was then used to synthesize cDNA. The sense and antisense primers for *EgHR3-like* were 5′-TGGCAGCGACACTACCTTTA-3′, 5′-AGTTCGCCTTGTTTCCCTTG-3′, and primers for *Eg-eif* (*E. granulosus* eukaryotic translation initiation factor) as an internal control [60] were 5′-GGGTAGAGAAATACATGCCATTG-3′ and 5′-TTCATCACTAACAGCGGAAGG-3′. The conditions used for real-time PCR were 95°C for 15 min, 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s for 40 cycles; the reaction was terminated by cooling to 4°C. All samples were run in triplicate. A SYBR Green PCR Kit (Qiagen, Germany) was used for the PCR and the reaction was conducted in an iCycler iQ™ system (BIO-RAD), with data analyzed according to the $2^{-\Delta\Delta Ct}$ method [15]. The efficiency of the primers has been tested and it has been confirmed no additional products are amplified. Statistical analysis used one-way ANOVA and Tukey's multiple comparison tests.

2.10. RNA interference (RNAi) assay

PSC were treated with pepsin and cultured in RPMI1640 medium containing fetal calf serum using the same protocols as described above. Negative control RNAi experiments were performed using either fluorescently labeled (Cy3-labeled negative control siRNA, Ribobio，GuangZhou, China) or non-labeled negative control small

RNA (Silencer negative control siRNA, Ribobio). The control RNAs without targets to any human, mouse, rat or *E. granulosus* genes were selected

For electroporation, 100 µl electroporation buffer containing approximately 2,000 PSC was placed in a 4-mm cuvette, and siRNA was added to give final concentrations of 1 µM or 5 µM. Pilot experiments indicated optimum electroporation occurred at 125 V–20 ms (Gene pulser, Bio-Rad) for the introduction of siRNA into PSC and this parameter was used in all subsequent experiments. After incubation at 37^{o}C for 10 min, the PSC in buffer were transferred into 1 ml culture medium and they were then further incubated at 37^{o}C in 24-well plates in the presence of 5% CO_2 in the dark. After 30 min and 2 h of silencing treatment, the PSC were washed with PBS and viewed under a Leica TCS SP8 Confocal laser scanning microscope to evaluate the efficacy of the treatment.

Four siRNA probes were used for the gene silencing analysis: non-labeled negative control siRNA (NsiRNA), siRNA1 (siRNA*EgHR3*-73), siRNA2 (siRNA*EgHR3*-304) and siRNA3 (siRNA*EgHR3*-373). The siRNA probes are 21 nt long, with 3' overhangs. The sequence of the siRNA1 is 5'-GGAUUCUUUAGACGGGCAUTT-3', the sequence of the siRNA2 is 5'-CCUGAGGACAGCAACCAAUTT-3', the sequence of the siRNA3 is 5'-CCAGGCACGAAUCUGUCAUTT-3'. After electroporation, the PSC were incubated at 37 ^{o}C for 10 min. The PSC were transferred into a 24-well plate with each well containing 1 ml culture medium. The plate was then incubated at 37^{o}C for a further 3 days in the presence of 5% CO_2. The experiment was carried out in triplicate. Untreated PSC were used as a control.

The RNAi effects on mRNA levels were evaluated using real-time PCR. For the analysis, total RNA was extracted, using TRIZOL reagent (Invitrogen), from all samples cultured at days 0 and 3 after electroporation. cDNA was then synthesized as described earlier (Section 2.9). Real-time PCR was carried out using 1µl of cDNA (1:5 dilution) and other components of the Qiagen (Germany) SYBR Green PCR Kit using the sameprimer sets as above. All samples were run in triplicate and underwent 40 amplification cycles at 95°C for 15 s and 60°C for 30 s using a StepOne Real-Time PCR System (Applied Biosystems) [37]. Each relative amount was normalized to the untreated control at day 0, and the data were analyzed according to the $2^{-\Delta\Delta Ct}$ method. Statistical analysis was then performed by one-way ANOVA and Tukey's multiple comparison tests.

The effects of siRNA (5µM) introduction by electroporation on the viability of PSC were evaluated in all cultured samples on day 3, 6, 10 and 15. Viability (%) was calculated by staining with 0.1% methylene blue [25] and counting the number of live PSC that were clear in appearance and contained transparent structures; dead PSC appeared opaque and had a rough surface and damaged inner structures. All samples were run in triplicate.

3. Results

3.1. Cloning and sequence analysis of EgHR3 -like

Sequence analysis showed that two predicted gene fragments of *E. granulosus* (GenBank accession no.EUB60779.1 and CDS18863.1)[57, 64] combine a full-length

of gene *EgHR3*. Based on the sequence, we designed primers to amplify the cDNA sequence of *EgHR3*-like by PCR. The full-length cDNA sequence obtained was identical to the two predicted sequences published recently(GenBank accession no.EUB60779.1 and CDS18863.1) [57, 64](data not shown). The cDNA has an open reading frame (ORF) of 1890 bp encoding 629 amino acids with a predicted molecular mass (MW) of 70 kDa and an isoelectric point (PI) of 9.09. Sequence analysis using BLAST and domain sequence alignment of EgHR3 with homologous sequences from other species showed that the amplified sequence contains a typical and conserved DNA-binding domain(DBD) which belongs to nuclear receptors (NR). The DBD is composed of two C4-type zinc fingers containing 8 conserved cysteines (Fig. 1). Residues 3–68 represent a zinc binding region. Motif scan analysis confirmed DBD at residues 3 to 74. In addition, the alignment showed the first zinc finger contains two conserved sequence regions ("CGD" and "CEGCKGFFRR") (Fig. 1). These conserved sequences may form the core for binding DNA. The DBD of EgHR3 was equipped with two zinc finger motifs, and an adjacent GRIP-box [12] with a "KLGRRS" sequence at C-terminal extension (CTE) to the DBD (Fig. 1).

EgHR3 also contains a moderately conserved ligand-binding domain (LBD) and a putative the ligand-dependent activation function domain 2 (AF-2) (Fig. 2), with a conserved "LYSETF" sequence.

3.2. Phylogenetic analysis

To further identify the evolution relationship of EgHR3 with other species, we

compared the DNA binding domain (DBD) of EgHR3 with this region of other 16 species (Table 1) including human (*Homo sapiens*, Hs), sheep (*Ovis aries*, Oa), mouse (*Mus musculus*, Mm), bird (*Tyto alba*, Ta), frog(*Xenopus laevis*, Xl), silkworm (*Bombyx mori*, Bm), fruit fly (*Drosophila melanogaster*, Dm) and worms (*Schistosoma haematobium*, Sh, and *Trichuris trichiura*, Tt). The phylogenetic analysis revealed that the HR3-DBD sequences formed three distinct clusters with *S. haematobium* located in a separated clade(Fig. 3). Supported by a high bootstrap value of 96%, EgHR3-DBD was most closely related to the HR3-DBD of oyster(*Crassostrea gigas*)(Fig. 3). The similar receptor of ROR (receptor-related orphan receptor)-beta-DBD of Chordata form a cluster, ROR-beta-DBD of Mammals (*Mm,Oa,Hs*) form one lineage. The HR3-DBD of Arthropoda form a cluster, HR3-DBD of insecta(*Dm,Aa,Bm,Td,Ln*) form one lineage, HR3-DBD of Crustacea(*Dp*) form one separated lineage(Fig. 3). The phylogenetic analysis further confirmed the isolated sequence is HR3 DBD.

3.3. Expression and purification of rEgHR3-DBD

EgHR3-DBD was successfully cloned into a pET vector and expressed in *E. coli* induced by IPTG. Fig. 4A shows the yield and size of the expressed protein on SDS-PAGE. The molecular mass of the affinity purified protein with His tag is about 37 kDa，which is coincident with the predicted size by bioinformatics analysis. A band corresponding to the expected molecular weight of EgHR3-DBD was present in the supernatant of cell lysates indicating the receptor protein is partially soluble in *E.coli*

culture medium under optimized culture conditions. The purified His-tag protein was recognized by an anti-His tag primary antibody in Western blot analysis (data not shown). To confirm the target EgHR3-DBD protein was expressed, we electrophoresed the recombinant protein purified by SDS-PAGE, stained and excised the band, sent it to BGI-Shenzhen for protein sequencing and the proteomic analysis confirmed the identity of the target protein sequence (data not shown).

3.4. Western blot analysis and immunolocalization of native EgHR3

Antiserum produced in mice against purified recombinant EgHR3-DBD induced an average serum titre of 1:500,000 determined by ELISA (data not shown). The antiserum was purified and used to probe native proteins isolated from different stages of *E. granulosus* by Western blot analysis. Figure 4B also shows a native protein band, with a molecular size of approximately 70kDa, in PSC and adult worms. The protein was highly expressed in PSC and adult worms. No signal was detected in hydatid cyst fliud. The Western blotting also shows that a native protein band, with a molecular size of approximately 35kDa, may indicate the degeneration of the native protein.

We used the affinity-purified anti-EgHR3-DBD antiserum to probe the parasite proteins *in situ* by immunofluorescence, this revealed that the EgHR3 protein was evenly distributed throughout all tissues of both the adult worms and scoleces (Fig. 5). No specific immunofluorescence was observed in either stage when probed with naive control serum (Fig. 5).

3.5. Transcription of EgHR3 in different development stages of E. granulosus

To identify whether the gene expression is regulated by bile acids, we cultured PSC developing adult worms in the medium containing bile acids. In the culture system, without bile acids, PSC develop into cysts. We then used real time PCR to determine the transcriptional levels of *EgHR3* in PSC (cultured with or without sodium taurocholate), adult worms and cyst membranes. The transcriptions were normalized with the transcription of *Eg-eif* as a house-keeping gene. Figure 6A shows that *EgHR3* was highly transcribed in adult worms, the transcription being 92- and 441-fold higher than in PSC and cyst germinal layer membrane, respectively.

To further examine the expression of *EgHR3* in early adult worm development, we compared the gene expression in PSC at different points with or without sodium taurocholate in the medium respectively. The highest level of *EgHR3* transcription occurred 3 hours after PSC were cultured with sodium taurocholate (PSC-B3h); it was 6.3 times higher than in PSC with PBS (‘normal’ PSC sample) or in PSC cultured for 3h without sodium taurocholate (Fig. 6B). No statistical differences were observed in the gene expression in PSC at different time points without sodium taurocholate.

3.6. RNAi silencing of the EgHR3 gene in E. granulosus *protoscoleces*

To determine whether silencing of *EgHR3* had an effect on parasite growth and development, we designed three small interfering RNA (siRNA) fragments based on the *Eg HR3* sequence and introduced the siRNAs into PSC. A pre-transformation assay using fluorescent dye labeled siRNA showed that PSC could be highly and efficiently transfected with small RNA by electroporation at 125V for 20 milliseconds (Fig. 7A).

We then transfected the three siRNAs into PSC in culture medium containing sodium taurocholate after PSC had been treated with pepsin. RT-PCR analysis revealed that, compared with PSC transfected with a negative control siRNA, the level of *EgHR3* transcription was reduced significantly ($P<0.01$) by 78% and 69% in PSC transfected with *EgHR3*-73 and *EgHR3*-373, respectively, at a concentration of 5μM siRNA after 3 days of culture (Fig. 7B). No statistical differences were observed between the NsiRNA and siRNA*Eg HR3*-304, indicating siRNA*EgHR3-304* had no inhibitory effect. A lower concentration (1 μM) of the siRNAs did not significantly change the level of *EgHR3* transcription.

We stained and counted live PSC at different time points after culture and calculated their viability as a measure of phenotypic change induced by the siRNA. There was no significant difference in viability on day 6 of any of the siRNA transfected PSC compared with PSC transfected with negative control siRNA ($P>0.05$). By day 10, however, there was 60.9% killing of PSC transfected with siRNA*EgHR3-73* compared with PSC transfected with control NsiRNA($P<0.01$). The order of killing effect or decrease of survival was siRNA*EgHR3-73*>siRNA*EgHR3-373*>siRNA*EgHR3-304* (Fig. 7C).

4. Discussion

Nuclear receptors (NRs) are ligand-regulated transcription factors that share a common domain architecture containing a DNA binding domain (DBD) with a highly conserved zinc-finger motif and a structurally conserved ligand binding domain (LBD) [6, 52]. We isolated HR3 from *E. granulosus*, which belongs to the NR1F superfamily.

NR1F is represented by ROR in vertebrates and CHR3 (aka, NHR-23) in *C. elegans* [53]. In the study, we identified *E. granulosus* HR3-like(EgHR3) based on the conserved structures including two zinc finger motif contains a group of 8 cysteine residues which form the core for binding the zinc ion. Beside the 8 cysteines, the conserved sequences "CGD" and "CEGCKGFFRR" are located in the first zinc finger (Fig. 1) of all the aligned sequences. The short conserved sequences may represent motifs forming the core of DNA binding sites. HR3 of *Drosophila melanogaster* (*Dm*) also contains a highly conserved C-terminal extension (CTE) to the DBD [12]. This CTE contains a "GRIP-box" sequence, which has the consensus sequence (K/R)XGRZ(P/S), where X is any amino acid and Z represents a hydrophobic amino acid[12, 33]. The GRIP-box typically provides stability to a monomeric receptor protein when binding DNA [63]. The EgHR3 DBD was equipped with two zinc finger motifs and an adjacent GRIP-box to the DBD, the GRIP-box sequence of EgHR3 is "KLGRRS"(Fig. 1). EgHR3 also contains a moderately conserved LBD and a putative AF-2 domain core (Fig. 2). The AF-2 consensus sequence of NR1F is LYXEZF, where X and Z are any amino acid. The AF-2 sequence of EgHR3 is "LYSETF" (Fig. 2). All these features indicate that the isolated sequence from E. granulosus is a *HR3*-like gene. Phylogenetic analysis of EgHR3-DBD also revealed that the isolated gene from E. granulosus was actually *HR3* (Fig. 3). Phylogenetic analysis revealed that all HR3-DBD formed three distinct clusters with Schistosoma haematobium located in a separated clade(Fig. 3). Supported by a high bootstrap value of 96%, EgHR3-DBD was most closely related to HR3-DBD from Crassostrea

gigas(Fig. 3). The close evolutionary relationship of HR3-DBD found in the Platyhelminthes with HR3-DBD of Mollusca is identical to their close phylogenetic relationship. The ROR-beta-DBD of Chordata form a cluster, ROR-beta-DBD of Mammalia(*Mm,Oa,Hs*) form one lineage. The HR3-DBD of Arthropoda form a cluster, HR3-DBD of insecta(*Dm,Aa,Bm,Td,Ln*) form one lineage, HR3-DBD of Crustacea(*Dp*) form one separated lineage(Fig. 3). The phylogenetic analysis revealed that all HR3-DBD orthologues are identical to from lower to higher phylogenetic relationship.

Ecdysone is best known as the insect molting hormone, it also has a role in vitellogenesis in the development of fruit fly and mosquito [5, 22]. Ecdysone has a function to affect microfilaria release in *Brugia pahangi* and meiotic reinitation in *D. immitis* [1]. Ecdysteroids were reported to be present in *Schistosoma* spp. and their level to vary during the development of post cercarial stages [55]. Evidence indicates that *S. mansoni* can synthesize ecdysone which plays a role in stimulating growth and vitellogenesis [41]. In *S. mansoni* miracidia, β-ecdysterone was reported to be effective in stimulating host location activities [47]. Ecdysone may play a role in strobilation (proglottid differentiation) of *M. expansa* [34]. In fact, ecdysteroids has been identified in the PSC of *E. granulosus* [36]. This suggests that the role of ecdysone in molting in Ecdysozoa lineage was acquired after the split of the Ecdysozoa and Lophotrochozoa. Thus, as the mode of action in insects, ecdysone and 20-OH ecdysone may play a key role in tapeworm development， including strobilation, detachment of mature proglottids, vitellogenesis and embryogenesis of

the tapeworm [36].

In insects, most effects of ecdysone are mediated through the heterodimeric Ecdysone receptor-Ultraspiracle receptor (ECR-USP)[19]. ECR is clearly the invertebrate orthologue of farnesoid X receptor (FXR); USP being the invertebrate orthologue of RXR[4, 53]. Ecdysone binding to its receptors directly regulates the transcriptional activity of the other 3 nuclear receptors ecdysone-induced protein 75(E75), HR3 and HR4. E75 is induced as a primary early response gene, while HR3 and HR4 are induced as early-late genes [19]. HR3 is induced after puparium formation, represses early genes [18, 19]. In Drosophila, HR4 acts with HR3 in the regulation of target genes, including FTZ-F1[18]. Therefore, cross-regulatory interactions between E75, HR3 and HR4 converge on FTZ-F1 to discriminate between the ecdysone responses of the first (puparium) and second (pupation) hormonal peaks that initiate the metamorphosis process[4]. In insects [14, 42] and some crustaceans [9], HR3 is induced by ecdysteroids and studies in insects have shown that HR3 mediates certain development-specific responses to ecdysteroid pulses, HR3 is a major component of ecdysteroid signaling and is under the negative regulatory control of E75 [12].

In Drosophila spp., HR39 is also active in ecdysone signaling [14, 56]. HR39 orthologue was known to be lost in certain lineages such as in chordates [3] while it was found in E. granulosus (GenBank: CDS15732.1) [57]. Full sequencing of Echinococcus spp. found genes encoding for downstream protein orthologue of ecdysteroid signal, including FTZ-F1 (GenBank: CDS15732.1), ecdysone-induced

protein 78C (GenBank: CDS17388.1) and ecdysone-induced protein 75B, isoforms C/D (GenBank: EUB64236.1)[57, 64]. HR3, FTZ-F1, ecdysone-induced protein 78C(E78C), and ecdysone-induced protein 75B(E75B) of *E. granulosus* share identity at the amino acid level with those of *Drosophila melanogaster* (GenBank: NP_788303.1, AAA28542.1, NP_524195.2, NP_730321.1) with 43%（50/117）, 59%（61/104）, 82%（68/83）, and 33%（127/390）similarity, respectively.

We showed that in the larval cystic stage of *E. granulosus EgHR3* is expressed at a very low level, more than 400 times less that in adult worms. The transcription level of *EgHR3* in adult worms (35days) was significantly higher than in normal PSC and the cyst germinal layer suggesting EgHR3 plays an important role in the growth and development of adult *E. granulosu*s. The precise involvement of EgHR3 in these processes remains to be defined, but could be partially explained by the recognized involvement of HR3 in cellular proliferation and mitogenic activity[1,12,]The level of transcription of *EgHR3* was higher in PSC cultured for 3 hours in the presence of bile acids(BA) than those non-BA stimulated PSC after treatment with pepsin and trypsin. This indicates that the mRNA expression of *EgHR3* was significantly increased under the stimulation by BA in the early stage of the development of the adult worm in *E. granulosus*. As the first time, we identified that the expression of EgHR3 was associated with bile acids, indicating that bile acid may be a ligand for EgHR3 or a stimulator for the HR3 and 20-hydroxyecdysone based molting cascade activation response in insects[[19, 4, 53]..

The silencing of *EgHR3* showed that this gene is crucial for the parasite. We used

two concentrations (1 μM and 5μM) of siRNA and the higher concentration inhibited transcription by 77.7%. We also found that gene silencing resulted in the death of 60% of PSC, further indicating *EgHR3* is critically important for parasite survival and thereby represents a novel drug target for *E. granulosus* definitive and perhaps also intermediate hosts, and vaccine candidate against adult worms of *E. granulosus* in dogs.

Summary of the ecdysone regulatory cascade, with the 11 transcription factors known to act as classic early regulators during the onset of Drosophila metamorphosis**(**Fig. 8B) [18, 19, 54]. The gene expression profile of *E. granulosus* suggests that upregulated genes have important roles in controlling and maintaining stage-specific features of the parasite during its life cycle[19]. It has been shown that BA have a crucial role in the differentiation of PSCs into adult worms, and E. granulosus may express BA transporters and nuclear hormone receptors to stimulate the relevant pathways(Fig. 8A)[19]. The mRNA expression of *EgHR3* was significantly increased under the stimulation of BA in the early stage of the development of the adult worm in *E. granulosus*, which suggests that BA can bind with FXR/RXR, BA-FXR/RXR complex then affects the transcriptional activity of EgHR3 to regulate adult development(Fig. 8A). In insects, most effects of ecdysone are mediated through the heterodimeric Ecdysone receptor-Ultraspiracle receptor (ECR-USP）[19]. ECR is clearly the invertebrate orthologue of farnesoid X receptor (FXR); USP being the invertebrate orthologue of RXR[4, 53]. FXR/RXR is clearly the orthologue of ECR-USP, as Ecdysone-ECR-USP complex regulates the

transcriptional activity of HR3, BA-FXR/RXR complex can regulates the transcriptional activity of HR3. FXR regulates gene expression by binding directly to DNA in vitro as a heterodimer with RXR or as a monomer [30, 46]. The FXR DNA-binding domain confers specific recognition of different DNA motifs called FXR response element (FXRE). In Drosophila, the promoter region of the HR3 gene contains four putative ecdysone response elements (ECRE) and is activated by 20E through a binding of ECR/USP complex to ECRE[45]. FXRE and ECRE can be homology, so BA-FXR/RXR complex can regulates the transcriptional activity of HR3 in *E. granulosu*(Fig. 8A). In Drosophila, ecdysone binding to its receptors directly regulates the transcriptional activity of HR3[45], as the action model of ecdysone , the ecdysteroids in protoscoleces of *E. granulosus* [36] can ultimately regulates the transcriptional activity of HR3(Fig. 8A). The orphan nuclear receptor HR3 is recognized as a central regulator in 20E-driven developmental switches during insect development and metamorphosis, and is responsible for directing timely shutdown of early genes regulated by a preceding 20E peak and a sequential activation of factors by a subsequent pulse of 20E[14]. As the similar action model of ecdysone in insects , EgHR3 can regulat adult development of E. granulosus. However, the detailed mechanism needs further study.

Acknowledgments

This work was funded by grants from the National Natural Science Foundation of China (U1303203) and Xinjiang Medical University Research Innovation Fund

Project of China (Grant No.XYDCX201423).We thank Mr Jiangli Yun and Mrs Suhui Liu for their technical support in tissue sectioning and staining.

References

1. Barker GC, Mercer JG, Rees HH , Howells RE. 1991. The effect of ecdysteroids on the microfilarial production of Brugia pahangi and the control of meiotic reinitiation in the oocytes of Dirofilaria immitis. Parasitol Res, 77(1), 65-71.

2. Benayahu D. 1997. Estrogen effects on protein expressed by marrow stromal osteoblasts. Biochem Biophys Res Commun, 233(1), 30-5.

3. Bertrand S, Brunet FG, Escriva H, Parmentier G, Laudet V , Robinson-Rechavi M. 2004. Evolutionary genomics of nuclear receptors: from twenty-five ancestral genes to derived endocrine systems. Mol Biol Evol, 21(10), 1923-37.

4. Bonneton F, Chaumot A , Laudet V. 2008. Annotation of Tribolium nuclear receptors reveals an increase in evolutionary rate of a network controlling the ecdysone cascade. Insect Biochem Mol Biol, 38(4), 416-29.

5. Bownes M. 1982. Hormonal and genetic regulation of vitellogenesis in Drosophila. Q Rev Biol, 57(3), 247-74.

6. Cotnoir-White D, Laperriere D , Mader S. 2011. Evolution of the repertoire of nuclear receptor binding sites in genomes. Mol Cell Endocrinol, 334(1-2), 76-82.

7. Craig PS , Larrieu E. 2006. Control of cystic echinococcosis/hydatidosis: 1863-2002. Adv Parasitol, 61, 443-508.

8. Eckert J, Deplazes P, Craig PS, Gemmell MA, Gottstein B, Heath DD, Jenkins DJ, Kamiya M , Lightowlers M. 2001. Echinococcosis in animals: clinical aspects, diagnosis and treatment. WHO/OIE Manual on Echinococcosis in Humans and Animals: a Public Health Problem of Global Concern, Eds Eckert J, Gemmell MA, Meslin F-X, Pawlowski ZS. Paris, France: World Organisation for Animal Health and World Health Organisation. 72-140 pp.

9. El Haj AJ, Tamone SL, Peake M, Sreenivasula Reddy P , Chang ES. 1997. An ecdysteroid-responsive gene in a lobster - a potential crustacean member of the steroid hormone receptor superfamily. Gene, 201(1-2), 127-35.

10. Espinola SM, Ferreira HB , Zaha A. 2014. Validation of suitable reference genes

for expression normalization in Echinococcus spp. larval stages. PLoS One, 9(7), e102228.

11. Gu SH , Chow YSJ. 1996. Insect Physiol. 42, 625-632.

12. Hannas BR, Wang YH, Baldwin WS, Li YC, Wallace AD , LeBlanc GA. 2010. Interactions of the crustacean nuclear receptors HR3 and E75 in the regulation of gene transcription. General and Comparative Endocrinology, 167(2), 268-278.

13. Hoffmann J , Porchet M. 1984. Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones,Springer-Verlag, Berlin.

14. Horner MA, Chen T , Thummel CS. 1995. Ecdysteroid regulation and DNA binding properties of Drosophila nuclear hormone receptor superfamily members. Dev Biol, 168(2), 490-502.

15. Hu Y, Huang L, Huang Y, He L, Zhang F, Li W, Liang P, Li R, Sun J, Wang X, Liang C, Li X , Yu X. 2012. Molecular cloning, expression, and immunolocalization of protein disulfide isomerase in excretory-secretory products from Clonorchis sinensis. Parasitol Res, 111(3), 983-9.

16. Jaiswal K, Lopez-Guzman C, Souza RF, Spechler SJ , Sarosi GA. 2006. Bile salt exposure increases proliferation through p38 and ERK MAPK pathways in a non-neoplastic Barrett's cell line. American Journal of Physiology-Gastrointestinal and Liver Physiology, 290(2), G335-G342.

17. Karlson P. 1983. Why are so many hormones steroids? Hoppe-Seyler's Z. Physiol. Chem., 364, 1067-1087.

18. King-Jones K, Charles JP, Lam G , Thummel CS. 2005. The ecdysone-induced DHR4 orphan nuclear receptor coordinates growth and maturation in Drosophila. Cell, 121(5), 773-784.

19. King-Jones K , Thummel CS. 2005. Nuclear receptors - A perspective from Drosophila. Nature Reviews Genetics, 6(4), 311-323.

20. Kozlova T , Thummel CS. 2000. Steroid regulation of postembryonic development and reproduction in Drosophila. Trends Endocrinol Metab, 11(7), 276-80.

21. Kozlova T , Thummel CS. 2003. Essential roles for ecdysone signaling during Drosophila mid-embryonic development. Science, 301(5641), 1911-4.

22. Laffitte BA, Kast HR, Nguyen CM, Zavacki AM, Moore DD , Edwards PA.

2000. Identification of the DNA binding specificity and potential target genes for the farnesoid X-activated receptor. J Biol Chem, 275(14), 10638-47.

23. Lee MR, Kim YJ, Kim DW, Yoo WG, Cho SH, Hwang KY, Ju JW , Lee WJ. 2012. The identification of antigenic proteins: 14-3-3 protein and propionyl-CoA carboxylase in Clonorchis sinensis. Mol Biochem Parasitol, 182(1-2), 1-6.

24. Li C, Kapitskaya MZ, Zhu J, Miura K, Segraves W , Raikhel AS. 2000. Conserved molecular mechanism for the stage specificity of the mosquito vitellogenic response to ecdysone. Dev Biol, 224(1), 96-110.

25. Li J, Wu C, Wang H, Liu H, Vuitton DA, Wen H , Zhang W. 2014. Boiling sheep liver or lung for 30 minutes is necessary and sufficient to kill Echinococcus granulosus protoscoleces in hydatid cysts. Parasite, 21, 64.

26. Li T, Ito A, Pengcuo R, Sako Y, Chen X, Qiu D, Xiao N , Craig PS. 2011. Post-treatment follow-up study of abdominal cystic echinococcosis in tibetan communities of northwest Sichuan Province, China. PLoS Negl Trop Dis, 5(10), e1364.

27. Luo D , luo K. 2015. Diagnosis and management of adult cerebral Echinococcosis. . Journal of Xinjiang Medical University 38(2), 223-226.

28. M B, P B, R L, E I , M C. 2002. Demonstration of a Direct Stimulatory Effect of Bile Salts on Rat Colonic Epithelial Cell Proliferation. . Scandinavian Journal of Gastroenterology, 37(1), 88-94.

29. Mane-Padros D, Cruz J, Cheng A , Raikhel AS. 2012. A Critical Role of the Nuclear Receptor HR3 in Regulation of Gonadotrophic Cycles of the Mosquito Aedes aegypti. Plos One, 7(9).

30. Mazuy C, Helleboid A, Staels B , Lefebvre P. 2015. Nuclear bile acid signaling through the farnesoid X receptor. Cellular and Molecular Life Sciences, 72(9), 1631-1650.

31. McManus DP, Gray DJ, Zhang W , Yang Y. 2012. Diagnosis, treatment, and management of echinococcosis. BMJ, 344:e3866, e3866.

32. McManus DP, Zhang W, Li J , Bartley PB. 2003. Echinococcosis. Lancet, 362(9392), 1295-304.

33. Melvin VS, Roemer SC, Churchill ME , Edwards DP. 2002. The C-terminal extension (CTE) of the nuclear hormone receptor DNA binding domain determines interactions and functional response to the HMGB-1/-2 co-regulatory proteins. J Biol

Chem, 277(28), 25115-24.

34. Mendis AH, Rees HH , Goodwin TW. 1984. The occurrence of ecdysteroids in the cestode, Moniezia expansa. Mol Biochem Parasitol, 10(2), 123-38.

35. Mercer JG, Munn AE, Arme C , Rees HH. 1987. Analysis of ecdysteroids in different developmental stages of Hymenolepis diminuta. Mol Biochem Parasitol, 25(1), 61-71.

36. Mercer JG, Munn AE , Rees HH. 1987. Echinococcus granulosus: occurrence of ecdysteroids in protoscoleces and hydatid cyst fluid. Mol Biochem Parasitol, 24(2), 203-14.

37. Mizukami C, Spiliotis M, Gottstein B, Yagi K, Katakura K , Oku Y. 2010. Gene silencing in Echinococcus multilocularis protoscoleces using RNA interference. Parasitology International, 59(4), 647-652.

38. Mohammadzadeh T, Sadjjadi SM, Rahimi HR , Shams S. 2012. Establishment of a Modified in Vitro Cultivation of Protoscoleces to Adult Echinococcus granulosus; an Important Way for New Investigations on Hydatidosis. Iranian Journal of Parasitology, 7(1), 59-66.

39. Montpied P, Sobrier ML, Chapel S, Couderc JL , Dastugue B. 1988. 20-Hydroxyecdysone induces the expression of one beta-tubulin gene in Drosophila Kc cells. Biochim Biophys Acta, 949(1), 79-86.

40. Moro PL, Gilman RH, Verastegui M, Bern C, Silva B , Bonilla JJ. 1999. Human hydatidosis in the central Andes of Peru: evolution of the disease over 3 years. Clin Infect Dis, 29(4), 807-12.

41. Nirde P, Torpier G, De Reggi ML , Capron A. 1983. Ecdysone and 20 hydroxyecdysone: new hormones for the human parasite schistosoma mansoni. FEBS Lett, 151(2), 223-7.

42. Palanker L, Necakov AS, Sampson HM, Ni R, Hu C, Thummel CS , Krause HM. 2006. Dynamic regulation of Drosophila nuclear receptor activity in vivo. Development, 133(18), 3549-62.

43. Palli SR, Hiruma K , Riddiford LM. 1992. An ecdysteroid-inducible Manduca gene similar to the Drosophila DHR3 gene, a member of the steroid hormone receptor superfamily. Dev Biol, 150(2), 306-18.

44. Palli SR, Ladd TR, Sohi SS, Cook BJ , Retnakaran A. 1996. Cloning and developmental expression of Choristoneura hormone receptor 3, an

ecdysone-inducible gene and a member of the steroid hormone receptor superfamily. Insect Biochem Mol Biol, 26(5), 485-99.

45. Riddiford LM, Hiruma K, Zhou X , Nelson CA. 2003. Insights into the molecular basis of the hormonal control of molting and metamorphosis from Manduca sexta and Drosophila melanogaster. Insect Biochemistry and Molecular Biology, 33(2003), 1327–1338.

46. Seol W, Choi HS , Moore DD. 1995. Isolation of proteins that interact specifically with the retinoid X receptor: two novel orphan receptors. Mol Endocrinol, 9(1), 72-85.

47. Shiff CJ , Dossaji SF. 1991. Ecdysteroids as regulators of host and parasite inter actions:a study of interrelationships between Schistosoma mansoni and the host snail,Biomphalaria glabrata. Tropical Medicine and Parasitology, 42((1), 11-16.

48. Siaussat D, Bozzolan F, Porcheron P , Debernard S. 2007. Identification of steroid hormone signaling pathway in insect cell differentiation. Cellular and Molecular Life Sciences, 64(3), 365-376.

49. Smyth J D. 1990. In Vitro Cultivation of Parasitic Helminths. 77–154 pp.

50. Smyth JD , Davies Z. 1974. In vitro culture of the strobilar stage of Echinococcus granulosus (sheep strain): A review of basic problems and results. Int J Parasitol, 4, 631-644.

51. Swevers L, Ito K , Iatrou K. 2002. The BmE75 nuclear receptors function as dominant repressors of the nuclear receptor BmHR3A. J Biol Chem, 277(44), 41637-44.

52. Thomas C, Pellicciari R, Pruzanski M, Auwerx J , Schoonjans K. 2008. Targeting bile-acid signalling for metabolic diseases. Nature Reviews Drug Discovery, 7(8), 678-693.

53. Thomson SA, Baldwin WS, Wang YH, Kwon G , LeBlanc GA. 2009. Annotation, phylogenetics, and expression of the nuclear receptors in Daphnia pulex. Bmc Genomics, 10.

54. Thummel CS. 2001. Molecular mechanisms of developmental timing in C. elegans and Drosophila. Dev Cell, 1(4), 453-65.

55. Torpier G, Hirn M, Nirde P, De Reggi M , Capron A. 1982. Detection of ecdysteroids in the human trematode, Schistosoma mansoni. Parasitology, 84(1), 123-30.

56. Truman JW, Riddiford, L.M. 2002. Endocrine insights into the evolution of metamorphosis in insects. Annual Review of Entomology, 47, 467–500.

57. Tsai IJ, Zarowiecki M, Holroyd N, Garciarrubio A, Sanchez-Flores A, Brooks KL, Tracey A, Bobes RJ, Fragoso G, Sciutto E, Aslett M, Beasley H, Bennett HM, Cai J, Camicia F, Clark R, Cucher M, De Silva N, Day TA, Deplazes P, Estrada K, Fernandez C, Holland PW, Hou J, Hu S, Huckvale T, Hung SS, Kamenetzky L, Keane JA, Kiss F, Koziol U, Lambert O, Liu K, Luo X, Luo Y, Macchiaroli N, Nichol S, Paps J, Parkinson J, Pouchkina-Stantcheva N, Riddiford N, Rosenzvit M, Salinas G, Wasmuth JD, Zamanian M, Zheng Y, Taenia solium Genome C, Cai X, Soberon X, Olson PD, Laclette JP, Brehm K , Berriman M. 2013. The genomes of four tapeworm species reveal adaptations to parasitism. Nature, 496(7443), 57-63.

58. Yao TP, Segraves WA, Oro AE, McKeown M , Evans RM. 1992. Drosophila ultraspiracle modulates ecdysone receptor function via heterodimer formation. Cell, 71(1), 63-72.

59. Zhang R, Gong J, Wang H , Wang L. 2006. Bile salts induce differentiation in cultured human normal esophageal mucosal epithelial cells. Zhonghua Yi Xue Za Zhi, 86(34), 2386-2390.

60. Zhang W, Li J, Jones MK, Z. Zhang LZ, Blair D , McManus DP. 2010. The Echinococcus granulosus antigen B gene family comprises at least 10 unique genes in five subclasses which are differentially expressed. PLoS Negl Trop Dis 4(8), e784.

61. Zhang W, Li J, You H, Zhang Z, Turson G, Loukas A , McManus DP. 2003. A gene family from Echinococcus granulosus differentially expressed in mature adult worms. Mol Biochem Parasitol, 126(1), 25-33.

62. Zhang W, Zhang Z, Wu W, Shi B, Li J, Zhou X, Wen H , McManus DP. 2015. Epidemiology and control of echinococcosis in central Asia, with particular reference to the People's Republic of China. Acta Trop, 141(Pt B), 235-43.

63. Zhao Q, Khorasanizadeh S, Miyoshi Y, Lazar MA , Rastinejad F. 1998. Structural elements of an orphan nuclear receptor-DNA complex. Mol Cell, 1(6), 849-61.

64. Zheng H, Zhang W, Zhang L, Zhang Z, Li J, Lu G, Zhu Y, Wang Y, Huang Y, Liu J, Kang H, Chen J, Wang L, Chen A, Yu S, Gao Z, Jin L, Gu W, Wang Z, Zhao L, Shi B, Wen H, Lin R, Jones MK, Brejova B, Vinar T, Zhao G, McManus DP, Chen Z, Zhou Y , Wang S. 2013. The genome of the hydatid tapeworm Echinococcus granulosus. Nat Genet, 45(10), 1168-75.

Table 1. Sequences used for alignment and phylogenetic analysis.

Protein ID	Protein Names	Species(abbreviation)	phyla	Nomenclature
	HR3-like	*E. granulosus(Eg)*	Platyhelminthes	NR1F
KGB37968.1	HR3	*Schistosoma haematobium(Sh)*	Platyhelminthes	NR1F
EKC29666.1	HR3	*Crassostrea gigas(Cg)*	Mollusca	NR1F
NP_001037012.1	HR3	*Bombyx mori(Bm)*	Arthropoda(Insecta)	NR1F
KMQ92824.1	HR3-like	*Lasius niger(Ln)*	Arthropoda(Insecta)	NR1F
AAF36970.1	HR3	*Aedes aegypti(Aa)*	Arthropoda(Insecta)	NR1F
ACY56691.1	HR3	*Daphnia pulex(Dp)*	Arthropoda(Crustacea)	NR1F
BAP76394.1	HR3	*Thermobia domestica(Td)*	Arthropoda(Insecta)	NR1F
NP_788303.1	HR3 isoform A	*Drosophila melanogaster(Dm)*	Arthropoda(Insecta)	NR1F
XP_014046834.1	ROR-beta	*Salmo salar(Ss)*	Chordata(Amphibia)	NR1F
XP_018109963.1	ROR-beta-like	Xenopus laevis(*Xl)*	Chordata(Amphibia)	NR1F
EMC87146.1	ROR-beta, partial	*Columba livia(Cl)*	Chordata(Aves)	NR1F
KFV42855.1	ROR-beta, partial	*Tyto alba(Ta)*	Chordata(Aves)	NR1F
KFU89045.1	ROR-beta, partial	Chaetura pelagic(*Cp)*	Chordata(Aves)	NR1F
NP_001036819.1	ROR-beta isoform 1	*Mus musculus(Mm)*	Chordata(Mammalia)	NR1F
NP_001123209.1	ROR-beta	*Ovis aries(Oa)*	Chordata(Mammalia)	NR1F
NP_008845.2	ROR-beta	*Homo sapiens(Hs)*	Chordata(Mammalia)	NR1F
CDW53506.1	HR3	*Trichuris trichiura(Tt)*	Nematoda	NR1F

Legends

Figure 1. Sequence alignment of DNA-binding domain (DBD) and its C-terminal extension**(**CTE**)** of *E. granulosus* EgHR3-like with them from other species . The conserved residues of DBD are highlighted with red. The conserved residues of CTE are highlighted with yellow. The conserved 8 cysteines of two C4-type zinc fingers are indicated with *. The GRIP-box in CTE is indicated by amino acid residues

KXGRZS in italics. Sequences used for alignment are in Table 1.

Figure 2. Sequence alignment of the ligand-binding domain (LBD) of *E. granulosus* EgHR3-like with LBD from other species. The two conserved motifs and the putative autonomous activation domain (AF-2) are highlighted in red. Sequences used for alignment are in Table 1.

Figure 3. phylogenetic analysis of the DBD sequence of *E. granulosus* EgHR3-like with DBD sequences of HR3/ROR from other species. Sequences used for phylogenetic analysis are in Table 1.

Figure 4. SDS-PAGE and Western blot analysis of EgHR3. SDS-PAGE analysis of recombinant His-EgHR3(rEgHR3), *E. granulosus* soluble protein extracts from adult worms (Adult), protoscoleces (PSC), cyst germinal layer membranes (CM) and hydatid cyst fluid (HCF) **(A)**. Western Blot analysis (using mouse anti-rEgHR3 antiserum). Protein markers are indicated in kDa **(B)**.

Figure 5. Immunolocalization of EgHR3 in protoscoleces (PSC) and adult worms of *E. granulosus*. EgHR3 in PSC. a and e, showing normal structure of PSC; b and f, stained with DAPI; c and g, probed with normal mouse serum and anti-EgHR3 antiserum, respectively; d and h, combined a, b and c, or e, f and g, respectively, showing co-localization of nucleus and EgHR3**(A)**. EgHR3 in adult worms of *E. granulosus*. i and m, showing normal structure of adult worms; j and n, stained with

DAPI; k and o, probed with normal mouse serum and anti-EgHR3 antiserum, respectively; l and p, combined i, j and k, or m, n and o, respectively, showing co-localization of nucleus and EgHR3**(B)**.

Figure 6. Quantitative real-time PCR analysis of the transcription of *EgHR3.*

The transcription of *EgHR3* at different developmental stages of *E. granulosus*. AW, adult worms; CM, cyst germinal layer **(A)**. The transcription of *EgHR3* at different time points of cultured *E. granulosus* protoscoleces **(B)**. PSC, protoscoleces; PSC-P: PSC treated with pepsin; PSC-PT: PSC treated with pepsin and trypsin; PSC-B3h, PSC-B12h, PSC-B24h: PSC treated with pepsin and trypsin then cultured with sodium taurocholate for 3, 12, 24 hours; PSC-B3d: PSC treated with pepsin and trypsin then cultured with sodium taurocholate for 3 days; PSC-B2w: PSC treated with pepsin and trypsin then cultured with sodium taurocholate for 2 weeks. The data were normalized using *Eg-eif* as a housekeeping gene. *, significant difference compared with the expression level in PSC.

Figure 7. Silencing of *EgHR3* by siRNAs.

Efficiency of electroporation for transformation of control labeled siRNA into PSC. a and b, untransfomed PSC; c and d, transformed PSC **(A)**. Silencing efficacy of siRNA for *EgHR3* in PSC at two concentrations (1 μM and 5 μM) of siRNA. U-0day and U-3day, untreated PSC or untreated PSC cultured *in vitro* for 3 days; E-3day, PSC treated by electroporation only and then cultured for 3 days; siRNA1-3, siRNA*EgHR3*-73, siRNA*EgHR3*-304 and siRNA*Eg HR3*-373 transfected PSC which

were cultured *in vitro* for 3 days; NsiRNA-3day, non-labeled negative control siRNA transfected PSC which were cultured *in vitro* for 3 days**(B)**. Silencing efficacy of small RNA fragments on the viability of *E. granulosus* PSC **(C)**.

Figure 8. Summary of the ecdysone regulatory cascade.

Summary of the putative regulatory cascade of E. granulosus adult development **(A)**. Bile acid (BA) is transported into cytoplasm, and then goes into nucleus, where it binds FXR/DVR/RXR to stimulate cell proliferation and growth. BA-FXR/RXR complex or EC can also regulate the transcriptional activity of EgHR3 to regulate adult development. Summary of the ecdysone regulatory cascade, with the 11 transcription factors known to act as classic early regulators during the onset of Drosophila metamorphosis**(B)** [18, 19, 54]. Large black bonds indicate the known protein–protein interactions.