International Journal for Parasitology 43 (2013) 641–646

Contents lists available at SciVerse ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

RNA-mediated gene suppression and in vitro culture in *Hymenolepis microstoma*

Natalia N. Pouchkina-Stantcheva [a], Lucas J. Cunningham [a], Gabriela Hrčkova [b], Peter D. Olson [a,*]

[a] Department of Zoology, The Natural History Museum, Cromwell Road, London SW7 5BD, United Kingdom
[b] Institute of Parasitology, Slovak Academy of Sciences, Hlinkova 3, 040 01 Košice, Slovak Republic

ARTICLE INFO

Article history:
Received 6 November 2012
Received in revised form 14 March 2013
Accepted 17 March 2013
Available online 29 April 2013

Keywords:
Hymenolepis
RNAi
In vitro cultivation
Platyhelminthes
Hox *Post-2*

ABSTRACT

Hymenolepis microstoma, the mouse bile-duct tapeworm, is a classical rodent-hosted model that provides easy laboratory access to all stages of the life cycle. Recent characterisation of its genome has greatly advanced its utility for molecular research, albeit contemporary techniques such as those for assaying gene function have yet to be developed in the system. Here we present research on the development of RNA-mediated gene suppression via RNA interference (RNAi), and on in vitro culture of the enteric, adult phase of the life cycle to support this work. We demonstrate up to 80% quantitative suppression of a Hox transcript via soaking activated juvenile worms with double-stranded RNAs. However, we were unable to achieve segmentation of the worms in culture despite extensive manipulations of the culture media and supplements, preventing functional interpretation. An alternative, in vivo approach to RNAi was also tested by exposing cysticercoids prior to inoculation in mice, but fluorescent labelling showed that the RNAs did not sufficiently penetrate the cyst body and no difference in expression was found between exposed and control groups grown in vivo. Genomic and transcriptomic data revealed that *H. microstoma* has two orthologs each of Dicer, Drosha and Ago-1-like genes and that expression of one of the Ago-1 genes appears exclusive to germline development, suggesting that two or more independent RNA-mediated pathways are in operation. These studies demonstrate the viability of RNAi in *H. microstoma* and extend the utility of the model for research in the genomic era.

1. Introduction

Cestodiases remain a significant threat to our health and agriculture and are gaining new ground due to climate change and globalisation (Davidson et al., 2012). Species of *Taenia* and *Echinococcus* are responsible for the most significant human infections, but are limited as laboratory models due to the practical inability of maintaining adult worms in vivo. *Hymenolepis microstoma* (Dujardin, 1845) Blanchard, 1891, the mouse bile-duct tapeworm, is a classical rodent-hosted model system in cestodology that provides easy laboratory access to the complete life cycle utilising natural definitive hosts that are themselves model organisms (i.e. mice and flour beetles). The utility of the *Hymenolepis* model has been advanced recently through whole genome and transcriptome characterisation (Holroyd and Sanchez-Flores, 2012; Olson et al., 2012; Tsai et al., 2013), description of the most widely employed laboratory strain (Cunningham and Olson, 2010) and ongoing studies on the content and expression of their developmental genes (Pouchkina-Stantcheva et al., 2011; Riddiford and Olson, 2011).

Reverse genetic techniques for assaying gene function such as RNA interference (RNAi) have not been previously applied to *Hymenolepis* spp. and require robust in vitro cultivation methods to support their development. The use of RNAi in flatworm research has to date had its greatest impact in the planarian model system in which ease of culture and robust protocols have led to a growing number of significant discoveries in recent years concerning the genetic control of their development (e.g. Dvořáková et al., 2004; Forsthoefel and Newmark, 2009). Among parasitic flatworms, a functional RNAi pathway has been demonstrated in the bloodfluke *Schistosoma* (Boyle et al., 2003; Skelly et al., 2003), in a monogenean (Ohashi et al., 2007) and most recently in tapeworms (Pierson et al., 2009; Mizukami et al., 2010; Spiliotis et al., 2010), indicating that RNA-mediated gene regulation is likely to operate throughout the phylum. At present, RNAi has been demonstrated in only a handful of helminth species and has been slow to progress toward becoming a routine and reliable method (Morales et al., 2008). It is also increasingly evident that not all genes can be targeted in this way; Pierson et al. (2009) demonstrated a working RNAi pathway in the ruminant cestode *Moniezia expansa*, but found that whereas abundantly expressed transcripts such as actin could be suppressed by delivering double-stranded (ds)RNA via soaking with or without electroporation, the neuropeptide transcripts of interest proved refractory. Most recently, RNAi has been applied to the fox tapeworm *Echinococcus multilocularis* (Mizukami et al., 2010; Spiliotis et al., 2010) through

* Corresponding author. Tel.: +44 207 942 5568; fax: +44 207 942 5151.
E-mail address: P.Olson@nhm.ac.uk (P.D. Olson).

0020-7519/$36.00
http://dx.doi.org/10.1016/j.ijpara.2013.03.004

the development of axenic culture methods for cystic stages of the parasite (Spiliotis and Brehm, 2009; Brehm, 2010b; Spiliotis et al., 2010). The *Echinococcus* system currently represents the most advanced model for the genetic manipulation of tapeworms, while also benefiting from a long history of molecular research on *Echinococcus* spp. Unfortunately, maintaining strobilate stages of these species in the laboratory cannot be done using common laboratory animals (i.e. rodents).

Studies on the laboratory cultivation of *H. microstoma* were conducted in the 1960s and 70s, during which the entire life cycle, from egg to gravid adult, was demonstrated in vitro (de Rycke and Berntzen, 1967; Evans, 1970; Seidel, 1975). Subsequent to these studies, however, we are unaware of any literature making use of their protocols, and therefore such practical tools in the *Hymenolepis* model system are now out of date. Here we concentrate on adult development in vitro, testing commercially available culture media and supplements on the growth of juvenile worms in order to support the development of RNAi. We used this as a platform for delivery of ds/short-interfering (si)RNAs and demonstrated quantitative suppression of a Hox transcription factor by up to 80%, but were unable to assess loss-of-function phenotypes as the cultures produced limited growth without the onset of strobilation. To circumvent the need for prolonged culture in vitro, an alternative approach was assessed involving in vitro delivery of dsRNA/siRNAs to cysticercoids that were then inoculated into mice and allowed to develop in vivo. However, effective delivery of dsRNAs to encysted, juvenile worms posed a problem, and further experiments with labelled siRNAs demonstrated that there was insufficient penetration of dsRNAs through the cyst body. We discuss alternative approaches to conducting RNAi in this model system and mine genomic and transcriptomic data to examine the components and expression of the RNAi machinery. Together with recent advances in the understanding of their genome (Tsai et al., 2013) and developmental genes, this work extends the utility of the *Hymenolepis* model system for contemporary research.

2. Materials and methods

2.1. Hymenolepis microstoma maintenance and collection

Stock cultures of the Nottingham strain of *H. microstoma* (see Cunningham and Olson, 2010) were maintained in vivo using outbred conventional BALB/c mice and flour beetles (*Tribolium confusum*). Larval stages were produced by exposing flour beetles for ~24 h to macerated, gravid tissues of *H. microstoma*, after which the tissues were removed and the beetles allowed to feed on flour ad libitum (see www.olsonlab.com for more information on in vivo maintenance of *H. microstoma*). Fully patent cysticercoids were collected from dissected beetles on or after day 7 post-exposure and washed twice for 30 min at room temperature (RT) in an antibiotic solution (20% PenStrep, 0.1% gentamicin in Earl's balanced salt solution (EBSS; Sigma–Aldrich, USA)).

2.2. Activation of cysticercoids and in vitro cultivation

Excystment of cysticercoids was performed according to a modified Rothman's procedure (Rothman, 1959) employing sterile conditions throughout. One hundred to 200 cysticercoids freshly harvested from the haemocoel of beetles were transferred to a falcon tube and soaked in sterile Ringer's solution with the addition of 1% pepsin and 1% HCl for 30 min at RT during which the juvenile worms became activated, evaginating their scolices and sloughing their cyst bodies. Activated juveniles were rinsed in sterile Ringer's solution and treated with 0.5% trypsin and 0.1% sodium taurochlorate (NaTC; bile salt) in EBSS for 20–25 min at 37 °C. The trypsin/NaTC solution was then diluted with an excess of EBSS to stop the enzymatic activity and the excysted worms incubated in EBSS containing 0.5% glucose, 5% Penicillin/Streptomycin (PenStrep) and 0.1% gentamicin for 15 min at 37 °C. Approximately 50 juvenile worms were transferred into 50 ml sterile culture flasks together with 10 ml of CMRL-1066 culture medium supplemented with 5% PenStrep, 20% heat inactivated foetal bovine serum (FBS), 0.5% glucose, 0.1% NaTC and 0.1% hemin. Apart from the CMRL-1066 and FBS culture media produced by Gibco (USA), all the other reagents were purchased from Sigma–Aldrich. Culture flasks also included an agar slant substrate made with culture media supplemented as above. A reducing atmosphere was created by replacing the ambient air in the flasks with a gas mixture of 95% N_2 and 5% CO_2. Flasks were capped and maintained on a gently oscillating rocker in an incubator at 39 °C. Cultures were monitored daily using an inverted light microscope and ~80% of the media replaced with fresh solution every 2–3 days.

During the course of the study, growth and survival of juvenile worms in each of three common basic media (supplemented as described herein) was compared: CMRL-1066, NCTC-135 and M199. In addition, we tested the effects of different concentrations of supplements to a basic media (i.e. NCTC-135), as shown in Table 1. No difference was observed in growth or mortality rates among the three basic media after 1 week, albeit the use of M199 appeared to result in slightly slower mobility and reduced growth (data not shown). Following these trials, NCTC-135 was used predominantly as the basic medium for subsequent assays.

Due to the site of attachment in the mouse host (i.e. bile duct), as well as the documented role of bile as a trigger for strobilation in some cestodes, the effect of whole bile on growth was also examined. Juvenile worms spend 2–3 days migrating in the small intestine prior to locating in the bile duct where they subsequently commence strobilation around day 6 p.i. (de Rycke, 1966). A range of tested regimes designed to test the effects of culturing in bile as a potential trigger for growth and strobilation, with exposure to basic, supplemented media before, during or after exposure to whole bovine bile, which was obtained from an abattoir and sterile filtered prior to use, are shown in Supplementary Table S1.

2.3. Detection of cell division using 5-bromo-2′-deoxyuridine (BrdU)

BrdU (5 mM, Sigma–Aldrich) was added to cultures in order to detect the presence of neoblast (i.e. stem cell) proliferation, and thus evidence of development in vitro. Worms grown in culture were exposed to BrdU for ~16 h, after which they were collected and fixed in freshly made 4% paraformaldehyde in PBS overnight at 4 °C. Detection was performed on whole mounts following the protocol of Koziol et al. (2010) with minor modifications. Specimens were mounted in Prolong Gold AntiFade reagent (Invitrogen, USA) and visualised using a Leica SP confocal microscope.

2.4. Preparation of ds/siRNAs

Long dsRNAs (385 bp) were produced using a MEGAscript® RNAi kit (Ambion, USA) following the manufacturer's instructions

Table 1
Effects of sodium taurochlorate, hemin and trypsin on the viability of juvenile *Hymenolepis microstoma* worms in vitro. All cultures (C1–C6) utilised NCTC-135 basic media supplemented with 5% PenStrep, 0.1% gentamicin, 20% foetal bovine serum and 0.5% glucose.

Culture	C1	C2	C3	C4	C5	C6
NaTC	0.1%	0.5%	1.0%	0.1%	0.5%	0.1%
Hemin	0.1%	0.1%	0.1%	0.5%	0.5%	0.1%
Trypsin	0	0	0	0	0	0.25%
Viability (days)	14	12	12	13	8	8

with primers encoding for the *Hymenolepis* posterior Hox ortholog *Post-2* (gene model HmN_000187500; http://www.genedb.org/Homepage/Hmicrostoma) and incorporating T7 promoter sites (shown in bold): Hm_*Post2*-T7/F (**TAATACGACTCACTATAGGGAG A**GGAGCCCTTTTGCGAGA) + Hm_*Post2*-T7/R (**TAATACGACTCACTAT AGGGAGA**CGTGTGATTGGGGTTGA). siRNAs were synthesised commercially by Dharmacon using the on-line siDESIGN centre tool (http://www.dharmacon.com/designcenter/designcenterpage.aspx). siRNA compositions were: *Post2*-ts37 (GGCAAGGAUCGGGUAG AAUUU) and *Post2*-ts57 (CAAAUCGUCUAGGUCCACCUU). The two siRNAs were then combined and used in subsequent experiments. The siRNA Wizard v3.1 tool (http://www.sirnawizard.com/scrambled.php) was used to generate a scrambled (i.e. non-coding) siRNA sequence (GUGGGAUAGAGGCGAAAUCUU) used as a control in all assays.

2.5. Quantitative gene expression analysis via real-time PCR (qPCR)

Purification of mRNA, cDNA synthesis and qPCR were performed as recently described by Pouchkina-Stantcheva et al. (2011). Cyclic AMP-dependent protein kinase (cAMP) was used as a reference gene for relative quantification as it has been shown to be expressed at consistent levels both spatially and temporally (Pouchkina-Stantcheva et al., 2011). qPCR primers were as follows: Hm-*cAMP*-QF1 (CGCCAAAGTGGTCAAAGGCCG) + Hm-*cAMP*-QR2 (GACCTGCAGGGCTTGATCCG), and Hm-*Post2*-QF2 (CCCCGCCA CCTCCTGGTCTT) + Hm-*Post2*-QR3 (TCCCCAGTCCTTCCGCCACC).

2.6. RNAi

2.6.1. In vitro assays

Activated juvenile worms produced as described in Section 2.2 were incubated in culture medium overnight at 37 °C with the addition of 0.5 μg/μl of dsRNA or siRNA. Control groups were incubated in medium only (dsRNA assays) or using scrambled siRNAs. After ~16 h the worms were removed from culture and frozen at −80 °C. qPCR analysis was performed as described in Section 2.5 on two independent in vitro assays for each control and experimental group. cDNAs were produced from a pool of ~100 juvenile worms from each group. In in vivo experiments ($n = 2$; see Section 2.6.2), cDNAs from six individual adult worms from each group (control and experimental) and each replica experiment, were quantified for relative *Post-2* expression.

2.6.2. In vivo assays

To circumvent the need for prolonged culture in vitro, the ability to generate RNA-mediated suppression during development in vivo was tested by first exposing cysticercoids to dsRNA in vitro and then inoculating them into mice. Approximately 300 cysticercoids freshly harvested from beetles were divided into control and experimental groups and pre-soaked in 100 μl of dsRNA solution (0.5 μg/μl dsRNA in PBS) or PBS without the addition of dsRNA (negative control) in eppendorf tubes overnight. Cysticercoids were transferred to 0.4 mm electroporation cuvettes in fresh dsRNA solution and electroporated at 100 V for 20 ms (single impulse square wave current) in 200 μl of dsRNA in RMPI-1640 (Sigma–Aldrich) (RMPI has been reported to be high performing medium for electroporation of schistosomes; Morales et al., 2008), then placed into RPMI medium for recovery and ~20 cysticercoids delivered via oral gavage to each of five mice.

2.7. Examining the efficiency of siRNA delivery

To determine the penetration of siRNAs introduced through soaking and electroporation of cysticercoid larvae, siRNAs were fluorescently labelled with the fluorescein derivative FAM (5′-carboxyfluorescein) using a Silencer siRNA labelling kit (Ambion) according to manufacturer's instructions. Cysticercoids were collected and washed in antibiotic solution as described in Section 2.1, transferred to 0.4 mm cuvettes in 200 μl of labelled siRNA solution (0.5 μg/μl of siRNA) and electroporated for 20 ms at 100 V (square wave). They were then mounted in Prolong Gold AntiFade reagent and visualised using a Leica SP confocal microscope.

3. Results and discussion

3.1. In vitro cultivation of H. microstoma

We followed previously reported methods (i.e. de Rycke and Berntzen, 1967; Evans, 1970; Seidel, 1971, 1975) for in vitro cultivation of the enteric phase of the *H. microstoma* life cycle as closely as currently available reagents and basic media formulations permitted. During the first 6 days in culture, the pattern of development was similar to that reported by Seidel (1975) and all excysted larvae had an active rostellum and good motility. Incorporation of BrdU (Fig. 1A) during this time demonstrated the presence of neoblast proliferation and thus active growth (see Koziol et al., 2010). Increased motility, development of the excretory ducts and an increase in length up to 1.5 times (compared with 16–18 h culture; Fig. 1B) were observed after 48 h (Fig. 1C). Growth proceeded until day 6 (Fig. 1D; 144 h in culture) with juvenile worms increasing in size up to 2.5–3 times the original size. However, we did not observe the onset of strobilation or the development of genital anlagen, and the worms thus remained juvenile. Continued cultivation in vitro (for up to a further 8 days) did not facilitate the beginning of strobilisation or further increase in size (data not shown), and mortality rates increased rapidly. In contrast, Evans (1970) reported the onset of internal strobilation in vitro by day 3 in some individuals, and external segmentation in 80–100% of individuals by day 7, while Seidel (1971) reported external segmentation and the formation of genital primordia in vitro by day 6.

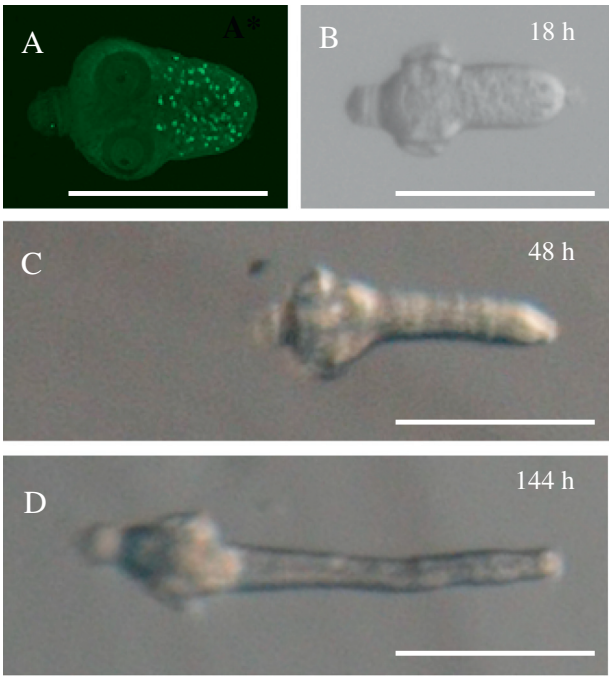

Fig. 1. In vitro cultivation of *Hymenolepis microstoma*. (A) 5-bromo-2′-deoxyuridine staining showing the presence of proliferating neoblasts (stem cells) at 16 h in culture. (B) Excysted larvae after 16–18 h in culture. (C) Juvenile worms after 48 h in culture. (D) Worms after 144 h in culture. Scale bars = 300 μm.

It is well appreciated that bile, or components therein, can act as a trigger for the onset of strobilation in at least some tapeworms, as has been demonstrated in vitro most recently in the fox tapeworm *Mesocestoides corti* (Saldaña et al., 2001; Markoski et al., 2003). Similarly, whole bile supplements (or components thereof) were found to be essential to the growth of *H. microstoma* in vitro (de Rycke and Berntzen, 1967). In addition to testing different concentrations of bile salt (NaTC; Table 1), we also tested the effects of pure bovine bile as a culture medium, in an attempt to mimic the unusual biology of *H. microstoma* which involves a migratory period of ~3 days in the upper small intestine before localising permanently in the bile duct (see Cunningham and Olson, 2010 for a detailed review of their life history). Strobilation then commences, at which time the worms are still too small (~3 mm) to extend into the lumen of the intestine and are thus bathed continuously in pure bile. Shortly thereafter they grow large enough to once again make contact with the small intestine and obtain nutrients from its contents. Therefore different regimes (Supplementary Table S1) were tested involving exposure to either pure or high concentrations (50% bile: 50% basic medium) of sterile-filtered bovine bile, either before or following growth in basic media supplemented as described in Section 2.2. However, in no instance did we observe the onset of strobilation, nor did pure bile appear to stimulate growth generally. Although the abiotic components of bile are highly similar among vertebrates, it is possible that bovine bile lacks secreted molecules specific to mice that could trigger strobilation in vivo. However, previous reports of successful development in vitro without the use of cell culture or mouse bile suggests that biotic factors are not required to trigger growth.

Seidel (1971) reported the necessity of hemin, an iron containing cofactor of haemoglobin, for the successful onset of strobilation. We believe, however, that the benefit of hemin was in acting as a reducing agent rather than a trigger for strobilation, as enteric helminths are highly susceptible to oxidative stresses in vitro. Although the onset of strobilation was not observed, our best culture results were obtained with the addition of 0.1% NaTC and hemin to the NCTC-135 basic media and agar substrate supplemented as described in Section 2.2. Further increases in concentration of NaTC led to slightly reduced viability (in C2 and C3 compared with C1; see Supplementary Table S1) and abnormal morphology was observed in cultures containing the highest concentration of NaTC (i.e. C3). Higher concentrations of hemin showed viabilities similar to C1, although this also led to visible deterioration of the tegument in some individuals. Larvae grown with the addition of trypsin (C4 and C5), a natural component of the rodent small intestine, showed the lowest viability and least growth among the cultures tested.

Remarkably, the literature details the successful cultivation of the complete *H. microstoma* life cycle in vitro, from egg to fully gravid adult (Seidel, 1975). In our hands, we were not able to achieve such success, albeit we have yet to try to reproduce the methods for culturing the larval phase. Larval development in vitro would not only provide a platform for experimental manipulation of this phase of the life cycle, but could also be a means by which to introduce foreign elements that could be carried into adult development in vivo and thereby support the ability to genetically manipulate adult as well as larval development. Nevertheless, available methods do provide for the activation and short-term culture of juvenile *H. microstoma* and a viable platform for the delivery of ds/siRNAs.

3.2. RNAi pathway components and expression in Hymenolepis

A number of RNAi pathways are present throughout the Eukaryota (Batista and Marques, 2011) and have recently begun to be characterised in parasitic platyhelminths (Gomes et al., 2009). The presence of multiple, distinct pathways in a single species appears to be common, if not the norm (Gomes et al., 2009). Mining the *H. microstoma* genome revealed two different orthologs of the RNase III proteins Drosha and Dicer, as well as two orthologs of the argonaute-like protein Ago-1 (see Table 2) that exhibit both PAZ and Piwi domains. Orthologs of these genes are also found in *Echinococcus* and *Taenia* spp. (Tsai et al., 2013), as well as in the bloodfluke *Schistosoma mansoni* (Gomes et al., 2009), suggesting that parasitic flatworms possess canonical, animal-specific RNAi pathways requiring processing by Drosha. Notably, however, true orthologs of the ubiquitous stem-cell markers Piwi and Vasa are not found in parasitic flatworms, indicating that their 'neoblast' stem cell system may be highly modified (Collins et al., 2013; Tsai et al., 2013).

Table 2
Expression of RNA interference pathway components in the *Hymenolepis microstoma* genome.

Gene	Gene model[a]	Position[a]	RNA-seq FPKM[b]								
			Sample[c]					Comparison[d]			
			Larvae	Adult	Scolex	Middle	End	Adult vs larvae	Middle vs scolex	End vs. scolex	End vs middle
RNase III proteins											
Dicer	HmN_000252400	Scaffold_27:510278-525458	28.1	23.1	10.4	14.4	5.3	ND	ND	DOWN	DOWN
	HmN_000200100	Scaffold_20:86953-97513	36.1	109.5	50.3	86.8	46.6	ND	ND	ND	ND
Drosha	HmN_000061600	Scaffold_5:252939-263252	8.8	25.6	11.5	23.5	9	ND	ND	ND	DOWN
	HmN_000200200	Scaffold_20:98426-107652	19.4	47.6	1	15.7	2.1	ND	UP	ND	DOWN
Argonaute-like proteins											
Ago-1-like	HmN_000114300	Scaffold_10:195110-201060	0.2	82.8	1.1	169.1	12.4	UP	UP	UP	DOWN
Ago-1-like	HmN_000658200	Scaffold_122:31264-45298	47.1	68.6	19.7	30.3	22.6	ND	ND	ND	ND

[a] Gene models and scaffolds available from http://www.genedb.org/Homepage/Hmicrostoma.
[b] FPKM (transcript fragments per kb of exon per million fragments mapped) values averaged across RNA-Seq replicates of each sample followed by statistical comparisons of differential regulation between samples.
[c] Larvae, mid-metamorphosing (i.e. 5 day old) larvae from the haemocoel of beetles; adult, whole adult worm from intestine of mice; scolex, scolex and neck region of the strobila; middle, middle region of strobila with sexually mature proglottides; end, end of strobila with gravid proglottides.
[d] ND, no difference in gene regulation for comparison; DOWN, down-regulation; UP, up-regulation.

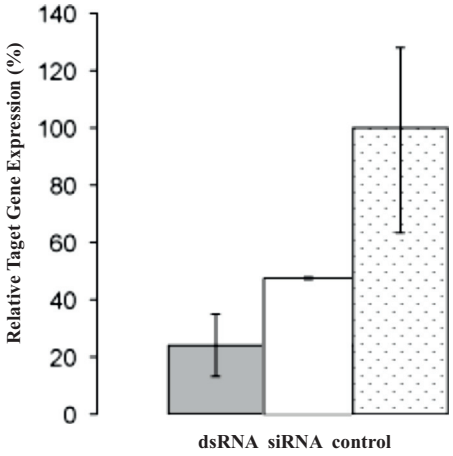

Fig. 2. RNA interference-induced suppression of Hox *Post-2* in vitro in *Hymenolepis microstoma*. Graph shows expression levels ($2^{-\Delta\Delta Ct}$ quantification) of *Post-2* relative to cyclic AMP-dependent protein kinase (cAMP) after soaking in double-stranded (ds)RNA, short-interfering (si)RNA or neither (control). Deviation bars indicate variability in gene expression levels between two trials.

Although RNAi has been used successfully in the laboratory for down-regulating target transcripts in both flukes and tapeworms, little is known about how the pathways differentially regulate gene expression during their complex life cycles. Table 2 shows RNA-seq expression levels of *H. microstoma* RNAi-associated genes stemming from a larger, ongoing study of tapeworm transcriptomics led by the Wellcome Trust Sanger Institute, UK. Data include expression levels for transforming larvae, mid-metamorphosis between the oncosphere and cysticercoid stages, for whole adults, and for three regions of the adult body: scolex/neck, mature segments and gravid segments. These data show that whereas one of the Ago-1-like orthologs is expressed throughout the life cycle, the other is primarily up-regulated in the region of the mature segments and therefore appears to be restricted to germline development, potentially substituting for the role of a true Piwi ortholog (see also Tsai et al., 2013). Ongoing gene expression studies in parasitic and free-living flatworms should provide a clearer picture of whether or not distinct RNAi pathways have become canalised to different roles, such as the maintenance of somatic vs germline stem cells.

3.3. RNAi in Hymenolepis

By soaking juvenile worms in ds/siRNAs we were able to suppress the expression of a Hox transcription factor up to 80% (Fig. 2). qPCR showed that RNAi-induced knockdown using long dsRNAs was more efficient than using siRNAs, which resulted in ~50% reduction. Although the in vitro system provided a platform for successful delivery of ds/siRNAs through soaking with or without electroporation, the limited growth achieved prevented us from determining the potential loss of function phenotype resulting from Hox suppression on strobilation and maturation. To circumvent this, an in vivo method was tested whereby cysticercoids were exposed to dsRNAs in vitro prior to infection of mice and then allowed to develop under natural conditions in vivo. Unlike the in vitro assays in which dsRNA was delivered to activated, juvenile worms, we predicted that excysted worms could not survive passage through the stomach, and therefore we attempted delivery of dsRNAs through soaking and electroporation of cysticercoids. However, after a period of 10–14 days development in vivo, worms recovered from both the experimental and control groups, appeared normal, were fully segmented and showed no quantitative difference in expression levels of the target gene (Fig. 3). Although we could not test expression levels prior to terminating infections, we did examine the efficiency of ds/siRNA delivery to the encysted juvenile worms using fluorescently labelled siRNAs. Fig. 3 shows confocal imaging of cysticeroids exposed to labelled siRNAs delivered through a combination of soaking and electroporation compared with those exposed to non-labelled siRNAs (control). Exposed cysticercoids showed evidence of siRNA uptake throughout the cyst body, but not in or around the encysted scolex of the juvenile worm, and cross-section reconstruction showed siRNAs had not penetrated into the juvenile worms and were lodged close to the surface of the cyst body (Fig. 3). The cyst body is moreover sloughed off upon activation of the juvenile worms and thus there is no further opportunity for the ds/siRNAs to penetrate once in the mouse host. It appears therefore that whereas soaking is a sufficient delivery method for excysted, juvenile worms, in which we expect both passive and active uptake through their unique tegument, this approach even when aided by electroporation does not yield sufficient penetration of the cyst body. A more targeted approach could prove more effective, such as microinjection through the cyst body directly into the juvenile worm. Such an approach has been piloted in our laboratory, but requires further investigation. Moreover, although an in vivo platform for RNAi may provide a viable alternative to in vitro culture, the latter is still desirable in order to provide a more controlled system for genetic manipulation, such as that discussed by Brehm (2010a).

Morales et al. (2008) recently reviewed current problems in the design and interpretation of RNAi studies in helminths and

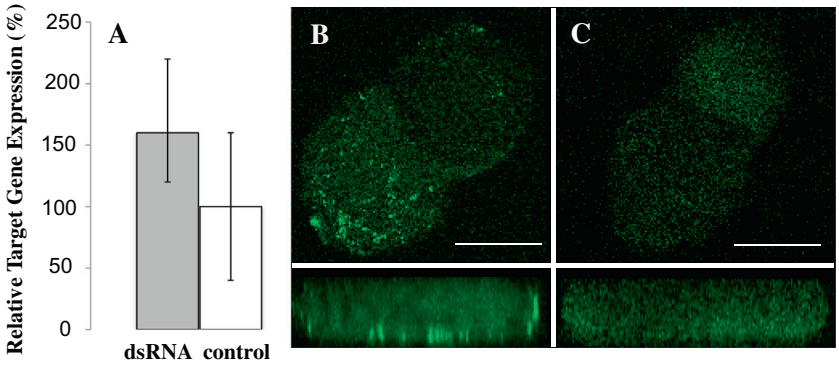

Fig. 3. In vivo RNA interference assays in *Hymenolepis microstoma*. (A) Comparison of Hox *Post-2* expression in adult worms cultured in vivo for 10–14 days following infection with cysticercoids either exposed to double-stranded (ds)RNA via soaking followed by electroporation or without exposure (control). Deviation bars show variability in *Post-2* expression between two independent experiments. Number of individual worms analysed for each group in each series, $n = 6$. (B) Confocal imaging of fluorescently-labelled short-interfering (si)RNAs showing their presence in the cyst body close to the surface following their delivery as described above. (C) Confocal image of a control cysticeroid exposed to non-labelled siRNAs. Scale bars = 100 μm.

provided extensive consideration of control measures to achieve more reliable and reproducible results. In most helminth model systems, however, robust culture and delivery methods remain among the principle problems in the development of RNAi, and as here, often prevent loss of function phenotypes from being assessed. Cestodes in particular lack a robust in vitro system for strobilar stages, which is no doubt complicated by the need to mimic the diverse environment of the vertebrate intestinal system, including not only gut contents but potentially host secreted factors as well. In vitro platforms supporting larval development of tapeworms such as the *E. multilocularis* system are by contrast well developed and support the screening of drug compounds against the ontogenetic stage of most relevance to our health, as well as allowing for transgenesis and other contemporary genetic manipulations to be introduced (Dvoroznáková et al., 2004; Brehm et al., 2006; Brehm and Spiliotis, 2008; Brehm, 2010a; Olson et al., 2012). The long history of research using *Hymenolepis* spp. combined with their suitability as models of adult development make it desirable to develop a similarly robust suite of tools to support contemporary research methods (Olson, 2008).

Acknowledgements

Special thanks to Magdalena Zarowiecki, Nancy Holroyd, Matt Berriman and the Wellcome Trust Sanger Institute, UK for producing genome and transcriptome data, and to Alejandro Sanchez-Flores for analyses of the RNA-Seq data. Thanks to Alex Gruhl and Alex Ball for assistance with confocal microscopy and to Murray Selkirk for use of his electroporator. Use of animals was approved and conducted in accordance with U.K. Home Office regulations under project license PPL 70/7150 granted to PDO. NPS and this work were supported by Biotechnology and Biological Sciences Research Council Grant BBG0038151 to PDO.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2013.03.004.

References

Batista, T., Marques, J., 2011. RNAi pathways in parasitic protists and worms. J. Proteomics 74, 1504–1514.

Boyle, J.P., Xiao-Jun, W., Shoemaker, C.B., Yoshino, T.P., 2003. Using RNA interference to manipulate endogenous gene expression in *Schistosoma mansoni* sporocysts. Mol. Biochem. Parasitol. 128, 203–215.

Brehm, K., 2010a. *Echinococcus multilocularis* as a model in stem cell research and molecular host–parasite interaction. Parasitology 137, 537–555.

Brehm, K., 2010b. The role of evolutionary conserved signalling systems in *Echinococcus multilocularis* development and host–parasite interaction. Med. Microbiol. Immunol. 199, 247–259.

Brehm, K., Spiliotis, M., 2008. Recent advances in the *in vitro* cultivation and genetic manipulation of *Echinococcus multilocularis* metacestodes and germinal cells. Exp. Parasitol. 119, 506–515.

Brehm, K., Spiliotis, M., Zavala-Góngora, R., Konrad, C., Frosch, M., 2006. The molecular mechanisms of larval cestode development: first steps into an unknown world. Parasitol. Int. 55, S15–S21.

Collins, J.J., Wang, B., Lambrus, B.G., Tharp, M.E., Iyer, H., Newmark, P.A., 2013. Adult somatic stem cells in the human parasite *Schistosoma mansoni*. Nature 494, 476–479.

Cunningham, L.J., Olson, P.D., 2010. Description of *Hymenolepis microstoma* (Nottingham strain): a classical tapeworm model for research in the genomic era. Parasite Vector 3, 123.

Davidson, R.K., Romig, T., Jenkins, E., Tryland, M., Robertson, L.J., 2012. The impact of globalisation on the distribution of *Echinococcus multilocularis*. Trends Parasitol. 28, 239–247.

de Rycke, P.H., 1966. Development of the cestode *Hymenolepis microstoma* in *Mus musculus*. Z. Parasitenkd. 27, 350–354.

de Rycke, P.H., Berntzen, A.K., 1967. Maintenance and growth of *Hymenolepis microstoma* (Cestoda: Cyclophyllidea) *in vitro*. J. Parasitol. 53, 352–354.

Dvoroznáková, E., Hrčkova, G., Borosková, Z., Velebny, S., Dubinsky, P., 2004. Effect of treatment with free and liposomized albendazole on selected immunological parameters and cyst growth in mice infected with *Echinococcus multilocularis*. Parasitol. Int. 53, 315–325.

Evans, W.S., 1970. The *in vitro* cultivation of *Hymenolepis microstoma* from cysticercoid to egg-producing adult. Can. J. Zool. 48, 1135–1137.

Forsthoefel, D.J., Newmark, P.A., 2009. Emerging patterns in planarian regeneration. Curr. Opin. Genet. Dev. 19, 412–420.

Gomes, M., Cabral, F., Jannotti-Passos, L., Carvalho, O., Rodrigues, V., Baba, E., Sá, R., 2009. Preliminary analysis of miRNA pathway in *Schistosoma mansoni*. Parasitol. Int. 58, 61–68.

Holroyd, N., Sanchez-Flores, A., 2012. Producing parasitic helminth reference and draft genomes at the Wellcome Trust Sanger Institute. Parasite Immunol. 34, 100–107.

Koziol, U., Dominguez, M.F., Marin, M., Kun, A., Castillo, E., 2010. Stem cell proliferation during *in vitro* development of the model cestode *Mesocestoides corti* from larva to adult worm. Front. Zool. 7, 1–12.

Markoski, M.M., Bizarro, C.V., Farias, S., Espinoza, I., Galanti, N., Zaha, A., Ferreira, H.B., 2003. In vitro segmentation induction of *Mesocestoides corti* (Cestoda) tetrathyridia. J. Parasitol. 89, 27–34.

Mizukami, C., Spiliotis, M., Gottstein, B., Yagi, K., Katakura, K., Oku, Y., 2010. Gene silencing in *Echinococcus multilocularis* protoscoleces using RNA interference. Parasitol. Int. 59, 647–652.

Morales, M.E., Rinaldi, G., Gobert, G.N., Kines, K.J., Tort, J.F., Brindley, P.J., 2008. RNA interference of *Schistosoma mansoni* cathepsin D, the apical enzyme of the hemoglobin proteolysis cascade. Mol. Biochem. Parasitol. 157, 160–168.

Ohashi, H., Umeda, N., Hirazawa, N., Ozaki, Y., Miura, C., Miura, T., 2007. Expression of vasa (vas)-related genes in germ cells and specific interference with gene functions by double-stranded RNA in the monogenean, *Neobenedenia girellae*. Int. J. Parasitol. 37, 515–523.

Olson, P., Zarowiecki, M., Kiss, F., Brehm, K., 2012. Cestode genomics – progress and prospects for understanding basic and applied aspects of flatworm biology. Parasite Immunol. 34, 130–150.

Olson, P.D., 2008. Hox genes and the parasitic flatworms: new opportunities, challenges and lessons from the free-living. Parasitol. Int. 57, 8–17.

Pierson, L., Mousley, A., Devine, L., Marks, N.J., Day, T.A., Maule, A.G., 2009. RNA interference in a cestode reveals specific silencing of selected highly expressed gene transcripts. Int. J. Parasitol. 39, 1–11.

Pouchkina-Stantcheva, N.N., Cunningham, L.J., Olson, P.D., 2011. Spatial and temporal consistency of putative reference genes for real-time PCR in a model tapeworm. Mol. Biochem. Parasitol. 180, 120–122.

Riddiford, N., Olson, P.D., 2011. Wnt gene loss in flatworms. Dev. Genes Evol. 221, 187–197.

Rothman, A., 1959. Studies on the excystment of tapeworms. Exp. Parasitol. 8, 336.

Saldaña, J., Marín, M., Fernández, C., Domínguez, L., 2001. In vitro taurocholate-induced sementation and clustering of *Mesocestoides vogae* (syn. *corti*) tetrathyridia (Cestoda) – inhibition by cestocidal drugs. Parasitol. Res. 87, 281–286.

Seidel, J.S., 1971. Hemin as a requirement in the development in vitro of *Hymenolepis microstoma* (Cestoda: Cyclophyllidea). J. Parasitol. 57, 566–570.

Seidel, J.S., 1975. The life cycle in vitro of *Hymenolepis microstoma* (Cestoda). J. Parasitol. 61, 677–681.

Skelly, P.J., Da'dara, A., Harn, D.A., 2003. Suppression of cathepsin B expression in *Schistosoma mansoni* by RNA interference. Int. J. Parasitol. 33, 363–369.

Spiliotis, M., Brehm, K., 2009. Axenic in vitro cultivation of *Echinococcus multilocularis* metacestode vesicles and the generation of primary cell cultures. Methods Mol. Biol. 470, 245–262.

Spiliotis, M., Mizukami, C., Oku, Y., Kiss, F., Brehm, K., Gottstein, B., 2010. *Echinococcus multilocularis* primary cells: improved isolation, small-scale cultivation and RNA interference. Mol. Biochem. Parasitol. 174, 83–87.

Tsai, I.J., Zarowiecki, M., Holroyd, N., Garciarrubio, A., Sanchez-Flores, A., Brooks, K.L., Tracey, A., Bobes, R.J., Fragoso, G., Sciutto, E., Aslett, M., Beasley, H., Cai, X., Camicia, F., Clark, R., Cucher, M., DaSilva, N., Day, T.A., Deplazes, P., Estrada, K., Fernández, C., Holland, P.W.H., Hou, J., Hu, S., Huckvale, T., Hung, S.S., Kamenetsky, L., Keane, J.A., 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, J.D., Zamanian, M., Zheng, Y., Consortium, T.T.s.G., Cai, J., Soberón, X., Olson, P.D., Laclette, J.P., Brehm, K., Berriman, M., 2013. The genomes of four tapeworm species reveal adaptations to parasitism. Nature 496, 57–63.