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Short technical report

Echinococcus multilocularis primary cells: Improved isolation, small-scale cultivation and RNA interference☆

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ABSTRACT

In this study we demonstrate RNA interference mediated knock-down of target gene expression in *Echinococcus multilocularis* primary cells on both the transcriptional and translational level. In addition, we report on an improved method for generating *E. multilocularis* primary cell mini-aggregates from *in vitro* cultivated metacestode vesicles, and on the cultivation of small numbers of small interfering RNA-transfected cells *in vitro* over an extended period of time. This allows assessments on the effects of RNA interference performed on *Echinococcus* primary cells with regard to growth, proliferation, differentiation of the parasite and the formation of novel metacestode vesicles *in vitro*.

The metacestode larval stage of the fox tapeworm *Echinococcus multilocularis* is the causative agent of alveolar echinococcosis, a serious zoonosis in rodents and humans [1]. Due to its accessibility to *in vitro* cultivation, *E. multilocularis* has during recent years been established as a laboratory model for studying the molecular basis of larval taeniid cestode development and host-parasite interactions [2]. The respective methodology comprises *in vitro* systems for the co-cultivation of *E. multilocularis* metacestode vesicles and host feeder cells, an axenic cultivation system for metacestode vesicles, and a system by which metacestode vesicles are generated *in vitro* from primary cell cultures in a manner that closely resembles initial parasite development from the oncosphere to the metacestode during natural infections (reviewed in Ref. [3]). In combination with the virtually completed *E. multilocularis* whole genome sequencing project and transcriptome approaches [2], these *in vitro* cultivation methods are suitable to initiate large-scale investigations on the role of defined parasite genes in *Echinococcus* larval development. However, apart from a method to transiently transfect cultivated *Echinococcus* cells [4], no methods have been established so far, that allow to knock-out or knock-down the expression of specific genes in this parasite.

During recent years, RNA interference (RNAi) has been established as a highly useful tool to inhibit the activity of targeted genes at the post-transcriptional level, thus mimicking the phenotype of 'loss of function' mutants [5]. Initially described for the nematode *Caenorhabditis elegans*, RNAi gene knock-down protocols have meanwhile been established for a number of flatworms such as free living planarians [6], trematodes [5], monogeneans [7] and the cestode *Moniezia expansa* [8]. In order to assess whether RNAi might also be applicable to *in vitro* cultivated *E. multilocularis* material, we first carried out genomic analyses on the current assembly version of the *E. multilcoularis* whole genome project (http://www.sanger.ac.uk/Projects/Echinococcus/; [2]), and scanned the data for the presence of components of the RNAi machinery. Two cellular factors, the double stranded RNA-specific ribonuclease dicer, and members of the argonaute-family of RNA binding proteins, which form the core component of the RNA-induced transcriptional silencing complex (RISC), have been identified as crucial components for RNAi in all systems investigated so far [9]. A dicer ortholog has recently been described in the related trematode *Schistosoma mansoni* [10] and by using this sequence as well as the sequences of dicer proteins from nematode and mammalian origin as a query in BLAST searches, one single ortholog could be identified in the *E. multilocularis* genome, located on contig 1698 of the current assembly version.

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers FN690740 (*emdicer*) and FN690741 (*emago*).

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Table 1
Sequence features and domain structure of EmDICER and EmAgo1.

	Position (aa)	Domain	Function	e-Value
EmDICER (1981 aa) (221 kDa)	18–236	DEXDc	Unwinding nucleic acids	<1e−12
	610–703	HELIc3	Unwinding nucleic acids	<1e−12
	833–956	Pfam:dsRNAbd	Binding of ds RNA	4.6e−4
	1184–1351	PAZ[a] domain	Complex formation with Piwi proteins	7.8e−8
	1504–1660	RIBOc domain	dsRNA-specific ribonuclease III	6.8e−17
	1711–1908	RIBOc domain	dsRNA-specific ribonuclease III	6.1e−33
	1914–1973	Pfam:dsrm	dsRNA binding	2.3e−2
EmAgo1 (881 aa) (99 kDa)	196–249	Pfam:DUF1785	Unknown; typical for Ago proteins	7.4e−23
	257–392	PAZ[a] domain	Complex formation with DICER	6.9e−5
	432–489	Piwi domain	dsRNA guided hydrolysis of ssRNA	<1e−12
	539–840	Piwi domain	dsRNA guided hydrolysis of ssRNA	2.8e−132

[a] Piwi, Argonaute, Zwille.

On the basis of the genome sequence, the entire mRNA sequence encoding the *E. multilocularis* dicer ortholog, EmDicer, could subsequently be PCR amplified in form of several overlapping fragments from metacestode cDNA preparations (data not shown). The EmDicer encoding mRNA comprised 6223 nt (excluding the polyA tail) and encoded a single protein of 1981 amino acids (theoretical molecular mass of 221 kDa) that displayed significant overall sequence homologies (between 25% identical and 40% similar residues) to known dicer proteins from *S. mansoni*, insects, nematodes and mammals (data not shown). In silico analyses using the simple modular architecture research tool (SMART; http://smart.embl-heidelberg.de/) further revealed that EmDicer displays a domain structure that is typical for the dicer family, including conserved DEXDc (DEAD-like helicase superfamily)-, double stranded RNA binding-, PAZ (piwi/argonaute/zwille)- and ribonucelase III-domains (Table 1). By RT-PCR analyses carried out on cDNA preparations, we could demonstrate that the EmDicer encoding gene is expressed in *E. multilocularis* primary cell cultures (after 1 week of cultivation; [4]) and in metacestode vesicles, as well as in invaginated and evaginated (low pH/pepsin-induced) protoscoleces (data not shown).

Humans express four different isoforms of argonaute of which only one, Ago2, is involved in mRNA decay and cleavage, whereas the other three isoforms mediate translational control [11]. We therefore used the human Ago2 sequence as a query to scan the *E. multilocularis* genome sequence for argonaute orthologs and obtained one clear hit (e-value of 3e−63) for contig 3006 as well as a locus with significantly lower homologies (4e−18) for contig 1632. On the basis of the considerable homologies of the locus on contig 3006, we decided to determine the respective mRNA sequence which was, again, achieved by RT-PCR amplification of overlapping fragments from cDNA preparations (data not shown). The entire cDNA of the respective gene, *emago*1, comprised 3261 nt (excluding the polyA tail) and encoded the argonaute-family protein EmAgo1 (881 amino acids; theoretical molecular mass of 99 kDa). On the amino acid sequence level, EmAgo1 displayed considerable homologies to known argonaute orthologs that are involved in RNAi such as human Ago2 (72% identical, 83% similar residues) or *Drosophila* argonaute 2 (32%, 49%), and possessed an overall domain structure that is typical for the argonaute protein family (Table 1). Like in the case of *emdicer*, *emago*1 was expressed in regenerating and mature metacestode vesicles as well as in invaginated and evaginated protoscoleces, as assessed by RT-PCR analyses (data not shown). By in silico analyses on the recently published genome of *S. mansoni* [12], several additional factors important for RNAi have recently been found [13,14] and in all cases when the schistosome sequences were used in BLAST analyses, corresponding factors were also identified on the *E. multilocularis* genome. This included orthologs to the ribonuclease Drosha, that is involved in the processing of primary miRNA transcripts (ortholog present on contig 2978), or a gene with considerable homology to SID-1 (contig 1610) which, in *C. elegans*, is necessary for systemic RNAi effects [14].

Having thus established that *E. multilocularis* contains and expresses the components necessary for systemic RNAi, we used our recently established system for the cultivation of parasite cells [4,15] to specifically knock-down target genes. However, employing primary *E. multilocularis* cell suspensions extracted under the previously described conditions, preliminary RNAi-experiments resulted in relatively low RNAi knock-down rates of 30–60% (data not shown). This was probably due to (i) the oxygen-sensitivity of primary cell cultures [16,4] and (ii) associated difficulties in handling these primary cells when using axenically grown metacestodes of the isolate H95 [17] as a source for parasite cells. To overcome these limitations, vesicles obtained from rat hepatoma co-cultures [3] were used for isolation of primary *E. multilocularis* cells. As a foothold, 1–2 ml of *in vitro* cultivated metacestode vesicles will provide isolated primary cells sufficient in number for one RNAi experiment using 100 μl sample in a 1 mm electroporation cuvette. Because low volumes of metacestode vesicles or primary cells are difficult to handle, we started with at least 30 ml of metacestode vesicles. In order to achieve quantitative removal of feeder cells, a two-step procedure was applied. First, additional washes of metacestode vesicles with PBS were introduced to the original protocol [4], resulting in the loss of a high number of hepatoma cells. Secondly, an overnight incubation step at 37 °C, 5% CO_2 in conditioned culture medium (cMEM = Dulbecco's MEM (Biochrom AG, Germany) + 10% non-heat inactivated FBS Superior (Biochrom AG) + 200 Units/ml PenStrep (Gibco BRL) conditioned for 1 week in the presence of 5×10^6 Reuber rat hepatoma cells and after sterile filtration supplemented with 10 μM bathocuproinedisulfonic acid (Sigma) [4]) resulted in quantitative adherence of hepatoma cells to the inner plastic surface of the well. The *E. multilocularis* primary cell mini-aggregates that had already formed from primary cells after this incubation step could be detached by cautious agitation. To achieve high-quality mini-aggregates, the isolated primary cells were seeded very densely into 6 well plates (amount of primary cells corresponding to 10 ml starting metacestode vesicles per well) and the mini-aggregates were centrifuged at low forces (2 min, 80 g) to remove damaged cells and debris that remained in the supernatant. A critical parameter was gentle handling of primary cells or mini-aggregates by pipetting very carefully. Furthermore, the centrifugation steps had to be performed at low speed (<300 g). As a consequence, the isolation procedure resulted in highly viable and basically feeder cell-free *Echinococcus* primary cell mini-aggregates, which were subsequently used for the RNAi-experiments.

Efficient RNAi knock-down on transcriptional and translational levels, as described below, could only be achieved by incorporating