Paving the way for transgenic schistosomes

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SUMMARY

In parasitological research, significant progress has been made with respect to genomics and transcriptomics but transgenic systems for functional gene analyses are mainly restricted to the protozoan field. Gene insertion and knockout strategies can be applied to parasitic protozoa as well as gene silencing by RNA interference (RNAi). By contrast, research on parasitic helminthes still lags behind. Along with the major advances in genome and transcriptome analyses e.g. for schistosomes, methods for the functional characterization of genes of interest are still in their initial phase and have to be elaborated now, at the beginning of the post-genomic era. In this review we will summarize attempts made in the last decade regarding the establishment of protocols to transiently and stably transform or transfect schistosomes. Besides approaches using particle bombardment, electroporation or virus-based infection strategies to introduce DNA constructs into adult and larval schistosome stages to express reporter genes, first approaches have also been made in establishing protocols based on soaking, lipofection, and/or electroporation for RNA interference to silence gene activity. Although in these cases remarkable progress can be seen, the schistosome community eagerly awaits major breakthroughs especially with respect to stable transformation, but also for silencing or knock-down strategies for every schistosome gene of interest.

Key words: Schistosomes, transgenesis, particle bombardment, electroporation, reporter genes, somatic transgenesis, germline transgenesis, RNAi.

INTRODUCTION

Today we are witness to remarkable progress in schistosome research. Supported by the improvement of automated sequencing technologies, comprehensive genome and transcriptome data have been generated (Oliveira *et al*. 2008; Berriman *et al*. 2009; The *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium, 2009). This marks the beginning of the post-genomic era, which necessitates the interpretation and exploitation of the amassed data. In this context it is essential to have technologies available allowing functional genetics. Heterologous expression systems such as *C. elegans*, mammalian cell lines or frog oocytes have been used to characterize parasite genes of interest in the past to overcome limitations associated with some parasite systems (Brooks and Isaac, 2002; Boyle and Yoshino, 2003; Britton and Murray, 2006). Although new data on gene regulation and functional evidence were obtained, heterologous systems are only partially able to provide indications for the function of a gene because it is expressed in a different genomic environment. Conclusive interpretations about gene functions can only be made upon its analysis in a homologous genomic environment. Therefore, it is of vital importance to establish transformation protocols for each parasite of interest.

For all plathyhelminths, transformation protocols have been developed demonstrating the possibility of generating genetically modified flatworms (Aboobaker and Blaxter, 2004; Grevelding, 2006; Spiliotis *et al*. 2008). The methods used are largely based on existing techniques and can be applied to any organism of interest if *in vitro* culture systems exist allowing the maintenance and manipulation of parasite life stages *ex vivo*. Premium prerequisites are met if accessible life stages can be repatriated into the life cycle, which is the case for schistosomes. To this end, miracidia can be harvested from eggs *in vitro*, transduced with nucleic acids by physical methods, such as particle bombardment (Wippersteg *et al*. 2002*a*,*b*, 2003), and used for snail infection afterwards (Beckmann *et al*. 2007). Furthermore, miracidia can be transformed *in vitro* and the emerging mother sporocysts transplanted into snails (Jourdane and Theron, 1980; Jourdane *et al*. 1985; Cohen and Eveland, 1988). Even daughter sporocysts can be generated *in vitro* from mother sporocysts (Bayne and Grevelding, 2003) and then transplanted into snails. Finally, schistosomula can be generated *in vitro* from cercariae and transplanted into final hosts such as mice (Nollen *et al*. 1976; Basch and Humbert, 1981; Clough, 1981). What approaches have been performed to generate transgenic schistosomes?

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SCHISTOSOME TRANSFECTION

In principle, several biochemical or physical methods are available for transfection and transformation. The most common ones are lipofection, microinjection or electroporation which were successfully used for a variety of cells or organisms including protozoan parasites (Clayton, 1999; de Koning-Ward *et al*. 2000; Meissner *et al*. 2007). Consequently, these methods were also tested in different laboratories to evaluate their potential for transient transformation of schistosomes.

In our laboratory, experiments with lipofection were performed with *in vitro* cultured adults or sporocysts and different commercially available lipofection reagents. Using plasmid-based DNA constructs with GFP (green fluorescent protein) as the reporter gene, no GFP fluorescence could be detected following lipofection regardless of the reagent used (K. Kapp, unpublished). In parallel, using fluorescence-labeled reagents for lipofection, it was shown that these reagents stick to the surface of adult schistosomes after treatment and no indications of uptake into subtegumental areas were observed (J. Kusel, Glasgow, 1999, personal communication). However, Nabhan and colleagues (2007) successfully used a lipofection reagent to introduce small interfering RNAs (siRNAs) into freshly transformed schistosomula. This indicates that either the stage used for lipofection or the combination of stage and reagent may be pivotal. The siPORT lipid transfection reagent (Ambion) used in their study had not been used by us before. Due to the soft structure of the tegument microinjection was found to be difficult. Approaches with adults and sporocysts showed the possibility to inject dyes which diffuse through the body after treatment (V. Wippersteg, unpublished). However, this technique is time consuming with a low efficiency. Only a very low number of individuals could be manipulated within one day, and most of these did not survive in culture afterwards. Electroporation was first successfully used by Correnti and Pearce (2004) introducing luciferase mRNA into *Schistosoma mansoni* schistosomula. The electroporation targeted the majority of the schistosomula, and immunolocalization studies indicated that the RNA was delivered to tegumental and subtegumental tissues. However, the RNA was unstable: luciferase activity declined by 24 h post-electroporation, and it was not detectable by 72 h. Nevertheless, these results opened the way to deliver DNA constructs as well as double stranded RNAs (dsRNAs) or siRNAs for RNA-silencing approaches (RNAi) into adult and larval schistosomes by electroporation. Shortly thereafter, electroporation was also established for transformation of *S. japonicum* (Yuan *et al*. 2005). In the following years, electroporation was used to introduce plasmid-based DNA constructs into schistosomula (Correnti *et al*. 2007; Morales *et al*. 2007). For adult schistosomes, electroporation was found to be inefficient for the introduction of DNA constructs, and this method may cause dysregulated transcription of reporter genes (Dvořák *et al*. 2010). Electroporation was also tested for miracidia but, depending on the conditions, miracidia either died or they were biologically inactive after treatment. Currently, electroporation is mainly used for the transient transformation of schistosomula with mRNAs or DNA constructs as well as for the delivery of dsRNAs and siRNAs into adult and larval *S. mansoni* or *S. japonicum*, but also into eggs of *S. mansoni* (Krautz-Peterson *et al*. 2007; Ndegwa *et al*. 2007; Zhao *et al*. 2008; Kines *et al*. 2010).

Finally, particle bombardment was also tested to transiently transform schistosomes, a biolistic approach that has been shown previously to work when other approaches had not. Particle bombardment was successfully used for different parasites including *Leishmania tarantolae* (Sbicego *et al*. 1998), *Brugia malayi* (Higazi *et al*. 2002), *Trypanosoma brucei* (Hara *et al*. 2002), and for the free-living nematode *C. elegans* (Wilm *et al*. 1999; Berezikov *et al*. 2004). Compared to microinjection, the biolistic approach allows the manipulation of a higher amount of individuals simultaneously and in a significantly shorter time period. One of the first reports on particle bombardment as a strategy to introduce nucleic acids in multicellular parasites was published more than a decade ago in a landmark study by Davis and colleagues (1999). They used embryos of the parasitic nematode *Ascaris* as a model to develop methods for the introduction of nucleic acids and then successfully applied these methods to adult schistosomes and introduced DNA constructs and mRNA. Using a plasmid containing the luciferase gene under the control of the *S. mansoni* spliced leader (SL)-RNA promoter, luciferase activity was found to be elevated 20-fold in adults after particle bombardment, indicating that the transgene is expressed in this organism. However, molecular or microscopical data demonstrating the level of transgene expression and the quality of the worms after bombardment were not provided for either *Ascaris* or for *S. mansoni*.

Particle bombardment

Parallel to the study of Davis *et al*. (1999) we had started a similar approach with the PDS 1000 particle bombardment system to transform adult and larval *S. mansoni*. In our experiments a modified form of the green fluorescent protein (GFP) gene from the jellyfish *Aequorea victoria* (Reichel *et al*. 1996) was used. Its expression generates strong visible bioluminescence (Chalfie *et al*. 1994), which can be easily detected by fluorescence microscopy. As

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regulatory elements to control the expression of the GFP reporter-gene we used promoter and terminator regions of a variety of known schistosome genes. Among these was the heat-shock protein (HSP) gene hsp70. Expression studies had demonstrated earlier that the schistosome hsp70 gene is developmentally regulated and inducible in adults by heat stress (Neumann *et al*. 1993). Thus a plasmid construct was made containing the GFP gene under the control of the promoter and terminator regions of the hsp70 gene. This construct was introduced by particle bombardment in adult *S. mansoni* males. Molecular analyses demonstrated the presence of the hsp70-GFP vector and its heat-inducible transcription and translation (Wippersteg *et al*. 2002*b*). Confocal microscopy finally confirmed correct transgene expression by exhibiting fluorescing signals in different areas of the worms that co-localized with the presence of gold particles. These signals were mainly found in the tegument and the tubercles of the males. Histochemical analyses by methylene-blue staining of 5 μm sections of bombarded worms indicated the presence of gold particles in nearly all tissues. Therefore, this specific signal localization was not due to an insufficient penetrance of gold particles into deeper tissue areas. Additionally, using an antibody against *S. mansoni* HSP70, its expression was detected predominantly in the tegument of worms after heat shock indicating a predominant role of HSP70 in the tegumental area following stress (Grevelding, 2006). Since the biolistic approach worked well with adults, it was also tried to perform bombardment experiments with larval schistosomes using sporocysts as targets. Establishing a more sensitive protocol with lower pressures assured the survival of the larvae after bombardment. Again, the presence and the transcription of the hsp70-GFP vector after bombardment were confirmed, and fluorescing signals were detected in different tissues of the sporocysts (Wippersteg *et al*. 2002*b*). Following up this work, a second 'proof of principle' vector was built consisting of GFP fused to the regulatory elements of the ER60 gene. It codes for a cysteine protease in schistosomes and was shown to be expressed in excretory/secretory tissues such as the gastrodermis or the protonephridia (Finken-Eigen and Kunz, 1997). After particle bombardment of adult schistosomes with an ER60-GFP vector, significant GFP signals were detected in the gastrodermis (Fig. 1 A, B). In addition, fluorescence was also observed as stripe-like structures within the parenchyma (Wippersteg *et al*. 2003). Using a colocalization approach of biolistic transformation with ER60-GFP and Texas Red (TxR)-BSA, a fluorescent dye that enters the excretory/secretory system and especially the excretory tubules as part of the protonephridium (Tan *et al*. 2003), the occurrence of GFP and TxR-BSA in the same tissue was confirmed (Wippersteg *et al*. 2003). Also in sporocysts, ER60 promoter-induced expression of GFP was localized in excretory/secretory tissues such as the lateral gland and the ridge cytons (Wippersteg *et al*. 2003). The same combination of biolistic transformation and co-localization with TxR-BSA was used by Rossi and colleagues (2003) to investigate the expression of calcineurin A from *S. mansoni*. Similar to the results of ER60, calcineurin A was also expressed in the excretory/secretory system of adult parasites. Using promoters of gut-specific genes it was later shown, in collaboration with James McKerrow's group at the University of California at San Francisco, that tissue-specific reporter gene activity can also be visualized in deeper tissue layers. To this end the promoters of the *S. mansoni* gut protease gene cathepsin L1 induced GFP expression in the gut following bombardment (Wippersteg *et al*. 2005). In the same study, the promoter of the protease gene cathepsin B2, whose protein product was known to be localized in the tegumental tubercles of males (Caffrey *et al*. 2002), was tested in a similar approach. Under the control of the regulatory elements of the cathepsin B2 gene fluorescence was observed in the tegument (Fig. 1 C, D), as expected, confirming the tissue-specificity of reporter gene expression after particle bombardment.

As well as particle bombardment works as a transformation method for adult and larval schistosomes, disadvantages are the relatively low number of transgenic worms after bombardment and/or the intensity of transgene expression in the parasite. To improve these parameters, Dvořák and colleagues (2010) recently modified the protocol. First, they created new constructs by fusing the GFP reporter gene with signal sequences of proteases and achieved tissue-specific GFP expression. Second, they introduced mCherry as new fluorescent reporter gene, which can serve as an alternative, spectrally distinct reporter besides GFP. However, mCherry signals were observed less frequently compared to GFP in the parasites and the rate of transgenic worms could not be increased. To consider electroporation as an alternative for the delivery of DNA constructs and transgenes into adult schistosomes, Dvořák *et al*. (2010) also tested this method. Their results indicated that electroporation, in contrast to particle bombardment, could lead to a non-specific expression of the reporter genes in adult schistosomes. Electroporation was also tested by Correnti *et al*. (2007) for schistosomules. Using the promoter sequence of the *S. mansoni* actin 1 (SmAct1.1) gene and luciferase as reporter, they were able to detect transgene expression in growing schistosomula (Correnti *et al*. 2007). In parallel, we tested the SmAct1.1 promoter to drive GFP expression in bombarded adult *S. mansoni* males and sporocysts (Beckmann *et al*. 2007). In adults, we detected GFP signals in the tegument including the tubercles, subtegument, parenchyma and in muscle cells (Fig. 1 E, F). This pattern corresponded perfectly to previous

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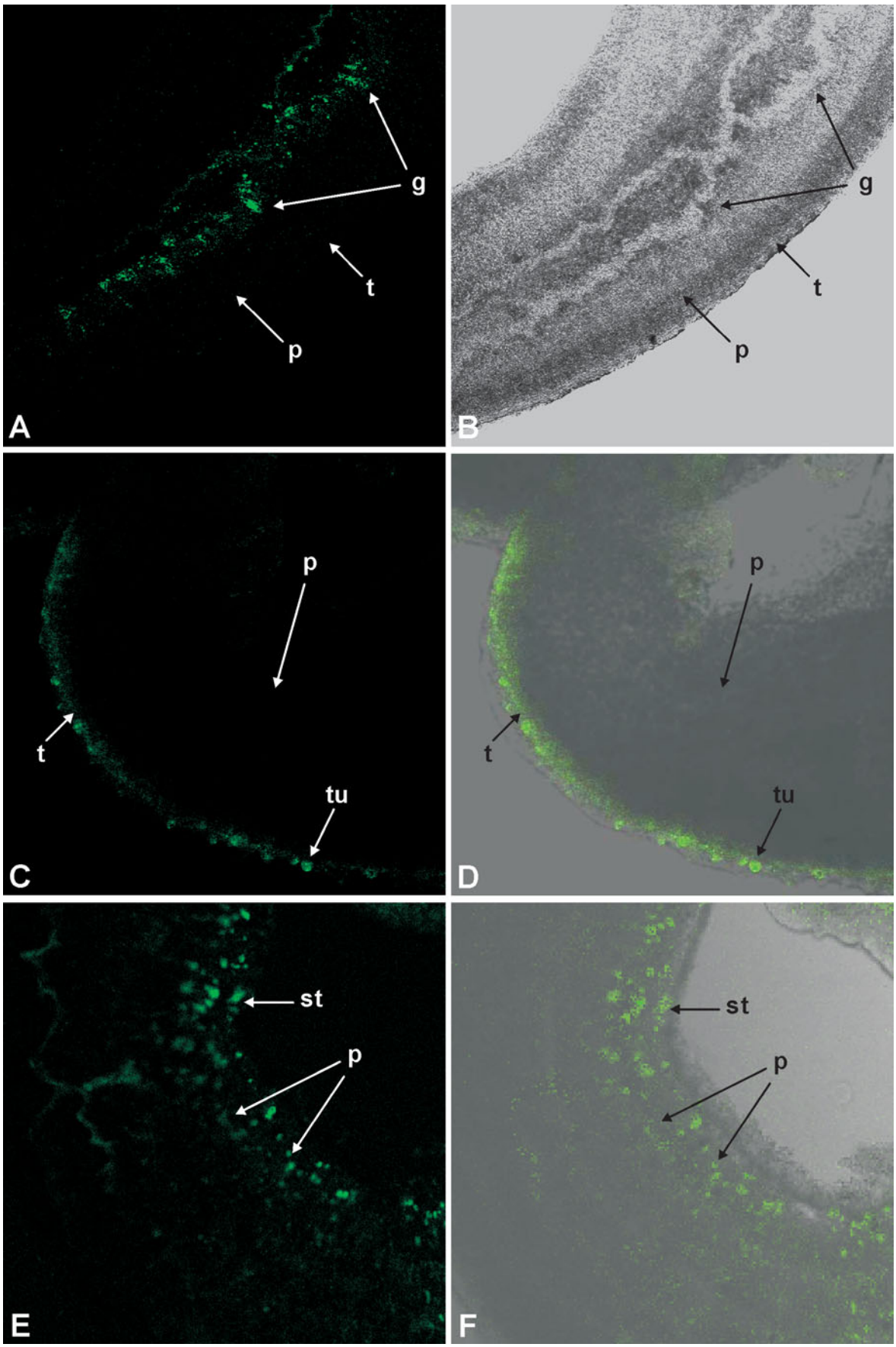

Fig. 1. For legend see next page.

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immunolocalization data showing the presence of actin in these tissues (MacGregor and Shore, 1990). Besides GFP and firefly luciferase, also a Gaussia luciferase was proven by Cheng and Davis (2007) in biolistic and electroporation experiments as a suitable reporter gene. The authors showed that, besides significantly higher levels of luciferase activity in schistosomes compared to other tested luciferases, the Gaussia luciferase can be secreted into culture media allowing non-invasive analysis of reporter gene activity (Cheng and Davis, 2007).

TRANSIENTLY TRANSFORMED SCHISTOSOMES

After the establishment of transient transformation in schistosomes, a number of studies have been performed using different regulatory elements and reporter genes introduced by particle bombardment (Davis *et al*. 1999; Wippersteg *et al*. 2002*a*,*b*, 2003, 2005; Heyers *et al*. 2003; Rossi *et al*. 2003; Beckmann *et al*. 2007; Dvořák *et al*. 2010; Table 1). All these studies proved the reliability of the method. However, the main disadvantage of this approach so far has been the transient nature of transgene expression. To further develop transgenesis in schistosomes, it has been necessary to explore methods for stable germline transformation which would allow genetic as well as phenotypic studies in subsequent generations. Towards this end, we developed a modified particle bombardment protocol that allowed the introduction of transgenes into the germline using miracidia as targets (Beckmann *et al*. 2007). After bombardment, the miracidia were still biologically active and able to infect snails to continue the life cycle – a critical step for the establishment and monitoring of stable transgenesis. As proofs of principle miracidia were bombarded with ER60-GFP-ER60 or hsp70-GFP-hsp70 constructs and afterwards used for the snail infection. Using molecular tools, we were able to detect the presence of the transgenes in cercariae and adults of the F_0 and the F_1 generation. These data demonstrated that transgenes can be passed on from one life stage to the next within one generation and, furthermore, from one generation to the next. Since the germ cells are considered to be the only constant cell line during schistosome development we could indirectly demonstrate the presence of transgenes in the germline and a successful germline-transformation approach (Beckmann *et al*. 2007). However, the presence of the transgenes could not be detected from generation F_2 on. Therefore, it seemed likely that the constructs occured extrachromosomally as episomes, which failed to integrate into the genome of the germ cells and thus were lost during cell divisions. In an independent study Heyers and colleagues (2003) confirmed the suitability of miracidia as starting stages for germ line transgenesis. In addition to demonstrating that bombarded miracidia could still infect snails, gold particles were detected in the germ balls of parasites within the snail tissue in which also reporter gene expression was also detected (Heyers *et al*. 2003).

FROM TRANSIENT TO STABLE TRANSFORMATION

Particle bombardment is a suitable method for adult and larval schistosomes to introduce vector constructs into the germline. However, strategies need to be developed that allow their integration into the genome of germ cells to achieve a heritable transgenesis. Approaches using mobile genetic elements as well as virus-based strategies have achieved the integration of transgenes into schistosome chromosomes, and the principle applicability of these approaches has been successfully shown (reviewed in Mann *et al*. 2008 and Hagen *et al*. 2011).

Mobile genetic elements – endogenous retroposons and DNA-transposons

Transposable elements (TEs) are subdivided into two groups according to their regulatory components and mechanistic features of their mode of action. TEs of class I represent retroposons which are generated and disseminated by reverse transcription of an RNA intermediate. Class II TEs transpose themselves or *via* a DNA copy (DNA-transposons). Depending on their physical integrity, class II TEs can be autonomous or non-autonomous with respect to their mobility. TEs are ancient vestiges of prokaryotic and eukaryotic genomes including those of parasites (Thomas *et al*. 2010; Venancio *et al*. 2010). In schistosomes, class I and II TEs have been described. Among the class I TEs are Boudicca and *fugitive*, *gypsy*-like long terminal repeat (LTR) retrotransposon or *Sinbad*, a Pao/BEL-type retrotransposon (Copeland *et al*. 2003, 2005). Molecular characterization of these TEs provided strong evidence for their

Fig. 1. Adult *S. mansoni* males following particle bombardment with the plasmid construct ER60-GFP-ER60 (A, B; Wippersteg *et al*. 2003), CB2-GFP-HSP70 (C, D; Wippersteg *et al*. 2005), or Act-GFP-Act (E, F; Beckmann *et al*. 2007) (A, C, E: fluorescence images; B: bright field image; D, F: overlay of fluorescence and bright field image). Fluorescence signals were detected by confocal laser scanning microscopy (Leica TCS NT) 24–48 hours after particle bombardment using a wavelength of 488 nm for excitation of GFP. The promoter regions of the cysteine protease gene SmER60, the cathepsin gene SmCB2, and the actin gene SmAct1 induced GFP expression as expected in the gastrodermis around the gut lumen (A, B), the tegument (C, D), and the subtegumental/muscle region as well as the parenchyma (E, F), respectively [g: gastrodermis, p: parenchyma, st: subtegument, t: tegument, tu: tubercle].

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Table 1. Approaches towards transgenic schistosomes (*S. mansoni, S. japonicum*)

Delivery method	Reference	Life cycle stage	Genetic material	Regulatory elements	Reporter gene	Activity/localization of reporter gene expression
particle bombardment	Davis *et al*. 1999	adult worms	plasmid DNA	SmSL promoter, enolase 3′ UTR and poly-adenylation signal	luciferase	luciferase activity was measured
			mRNA	–		
	Wippersteg *et al*. 2002*b*	adult males, sporocysts	plasmid DNA	SmHsp70 promoter and terminator	GFP	tegument surface several tissues
	Wippersteg *et al*. 2002*a*	sporocysts	plasmid DNA	SmER60 promoter and terminator	GFP	excretory/secretory system
	Wippersteg *et al*. 2003	adult males	plasmid DNA	SmER60 promoter and terminator	GFP	excretory/secretory system
	Rossi *et al*. 2003	adult males	plasmid DNA	SmCalcineurinA promoter and terminator	GFP	excretory/secretory system
	Heyers *et al*. 2003	adult males, miracidia	plasmid DNA	SmHsp70 promoter and terminator	EGFP	tegument surface (transfected miracidia infective for snails)
	Wippersteg *et al*. 2005	adult males	plasmid DNA	SmCL1 or SmCB2 promoter and SmHsp70 terminator	GFP	gut, tegument
	Beckmann *et al*. 2007	adult males, sporocysts, mircidia	plasmid DNA	SmAct1 promoter and terminator, SmHsp70 or SmER60 promoter and terminator	GFP	tegument, parenchyma, muscle cells of adults; several tissues of sporocysts; transfected miracidia infective for snails; transgenes were passed on to the next generations
electroporation	Correnti and Pearce, 2004	schistosomula	mRNA	–	luciferase	tegumental and subtegumental tissues
	Yuan *et al*. 2005	schistosome cells, schistosomula, adults	plasmid DNA	CMV promoter	EGFP	reporter gene expression was confirmed by molecular analyses and microscopically
	Correnti *et al*. 2007	schistosomula, immature schistosomes	plasmid DNA	SmAct1 promoter, SmSL promoter, SmMHC 3′ UTR, SmAct 3′UTR, SV40 3′UTR	luciferase	luciferase activity was measured
	Morales *et al*. 2007	schistosomula	piggyBac donor plasmid + transposase mRNA	SmAct1 and SmHsp70 promoter, SV40 3′UTR	luciferase	integration of the piggyBac transposon into genomic DNA
particle bombardment vs electroporation	Chen *et al*. 2007	miracidia, sporocysts, schistosomula, adults	mRNA	–	different luciferases	luciferase activity was measured
	Dvořák *et al*. 2010	adults, immature worms	plasmid DNA	SmCD or SmCF promoter and terminator	GFP mCherry	gut (particle bombardment) tissue-unspecific (electroporation)

retroviral transduction	Kines *et al*. 2006	schistosomula, sporocysts	VSVG-pseudotyped MMLV virions	SmSL or SmHsp70 promoter	EGFP	reporter gene expression was detected by RT-PCR
	Kines *et al*. 2008	schistosomula, adults	VSVG-pseudotyped MMLV virions	SmSL or SmAct1 promoter	luciferase	integration of retroviral provirus into genomic DNA; luciferase activity was measured and detected microscopically
	Rinaldi *et al*. 2011	adults, fragmented adults	VSVG-pseudotyped MMLV virions	SmAct1 promoter	luciferase	integration of retroviral provirus into genomic DNA
	Yang *et al*. 2010	schistosome cells, schistosomula	virions	viral LTRs	hTERT	reporter gene expression was detected by RT-PCR and immunolocalization
retroviral transduction + electroporation	Kines *et al*. 2010	eggs	mRNA, VSVG-pseudotyped MMLV virions	SmAct1 promoter	luciferase	luciferase activity was measured and detected microscopically; integration of retroviral provirus into genomic DNA

[EGFP: enhanced green fluorescent protein; GFP: green fluorescent protein; hTERT: human telomerase reverse transcriptase].

transcriptional activity in larval and adult stages of schistosome development, and they occur in different schistosome species. Therefore, it has been debated whether they may have the potential to become useful tools for the establishment of a retroposon-based transgenesis system (Mann *et al*. 2008). Additionally, the *Boudicca* and *Sinbad* LTRs were tested for promoter function, and it was shown that the *Sinbad* LTRs but not the *Boudicca* LTRs were able to drive transgene activity in human cell culture (Copeland *et al*. 2007).

Sm*Merlin* is a class II TE of *S. mansoni* and may represent another vehicle candidate for transgenesis. Due to its similarity to bacterial insertion sequences *Merlin* belongs to the superfamily of *Merlin*/IS1016 DNA elements, which occur also in higher eukaryotes. More than 500 Merlin sequences were identified in the genome of *S. mansoni*, many of these are deletion variants (Feschotte, 2004). This indicates that the *Merlin* family consists of autonomous and non-autonomous members. BlastN-analyses in EST databases of *S. mansoni* and *S. japonicum* resulted in many hits indicating not only transcriptional activity, but also the occurrence of *Merlin* in different schistosome species. Two kinds of transcripts were found, those with and those without flanking host sequences. This shows that *Merlin* family members can integrate in transcriptional active regions of the genome, but it also indicates a potential influence of transcriptionally active schistosome genes in the neighbourhood on the expression of *Merlin* family members. Consequently, typical Merlin footprints were identified in the genome of *S. mansoni* (Feschotte, 2004) consisting of 8 bp target site duplications, which occur during the integration process and remain after the transposon has left this target site again. All these results suggest that *Merlin* is an active mobile element tramping through the schistosome genome. Its relatively small size of 1·4 kb makes Sm*Merlin* a 'lab-friendly' candidate for vector construction. Sm*Merlin* has two exons separated by a 32 bp intron. The exons code for a protein of 294 amino acids which reveals significant homology to transposases (Feschotte, 2004). The ends of *Merlin* are characterized by 24 bp terminal inverted repeats. To start cloning vectors for transgenesis on the basis of Sm*Merlin* we amplified *Merlin* transposase sequences recently and detected that all of these contained the 32 bp intron (Beckmann *et al*., unpublished). Its sequence predicts that presence of this intron may lead to a premature stop of translation and an incomplete transposase. This finding indicates that Sm*Merlin* activity may be controlled in the schistosome genome in a similar way as known from P-elements in *Drosophila* (Bachmann and Knust, 2008). Here, an intron between the open reading frames 2 and 3 is not spliced in somatic cells, which prevents any mobilization, whereas in the germline splicing occurs leading to transposition in this cell

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line (Laski *et al*. 1986). By cloning *Merlin*-based constructs containing the *Merlin* transposase without the 32 bp intron it seems feasible to develop a potent transformation system. Using particle bombardment we were able to introduce these *Merlin* constructs into adult and larval schistosomes and the transcription of the transgene *Merlin* transposase was detected afterwards by RT-PCR analyses. In future studies we will focus on the detection of excision and integration events to evaluate this system for its suitability to stably transform schistosomes. Besides *Merlin*, a member of the CACTA (also called En/Spm) superfamily of DNA transposons was also described for schistosomes (DeMarco *et al*. 2006). It was called Sm*TRC1* and suggested to be a potential new tool for insertional mutagenesis because CACTA elements had been successfully used for this purpose in plants (Tissier *et al*. 1999). As for *Merlin*, future studies have to show whether Sm*TRC1* is another candidate for DNA transposon-based transformation.

Exogenous DNA-transposons

PiggyBac, a class II TE originating from the genome of the cabbage looper moth *Trichoplusia ni*, has been successfully used as a vehicle for transformation in diverse eukaryotic organisms such as mosquitoes, planarians, *Plasmodium falciparum*, but also in human or other mammalian cells. Morales and colleagues (2007) demonstrated that *piggyBac* is able to deliver reporter transgenes into the genome of *S. mansoni*. After electroporation of schistosomula with a *piggyBac* donor plasmid containing the firefly luciferase gene under the control of schistosome gene promoters together with *piggyBac* transposase mRNA, numerous transposon integrations into the parasites' chromosomes were detected. This result represented substantial progress in somatic transgenesis, although the inheritance of *piggyBac*-based vector constructs has yet to be demonstrated.

Virus-based approaches

Since retroviruses have been used successfully for transgenesis in other organisms, Kines and colleagues (2006) established a transduction system for *S. mansoni*, using replication incompetent Moloney Murine Leukaemia Virus (MMLV) virions that were pseudotyped with Vesicular Stomatitis Virus Glycoprotein (VSVG) carrying EGFP or luciferase reporter genes under the control of the Sl or hsp70 promoter. After co-cultivation of larval schistosomes (schistosomules or sporocysts) with the virions, the authors showed virus binding and uptake into the parasite tegument. Thus, the retroviruses seemed able to transduce cultured schistosomes. Finally, evidence of proviral integration into genomic DNA as well as the presence of transcripts encoding reporter transgenes were obtained. The same group later used Southern blot analysis and an anchored PCR-based approach to demonstrate integration of proviral MMLV retroviruses into schistosome chromosomes, proving somatic transgenesis. Furthermore, reporter gene/luciferase activity in transduced schistosomula and adult schistosomes was measured (Kines *et al*. 2008). The anchored PCR approach detects the transgenes in the chromosomes and also determines the efficiency of transduction after the exposure of schistosomes to virions (Rinaldi *et al*. 2011). Furthermore, the transduction approach with VSVG-pseudotyped retroviruses also works for schistosomules of *S. japonicum* (Yang *et al*. 2010). Besides larval and adult schistosomes, also *S. mansoni* eggs can serve as targets for VSVG-pseudotyped MMLV virions (Kines *et al*. 2010). After exposure of schistosomes eggs to virions by either soaking or electroporation, proviral transgenes were detected by PCR within the genomic DNA of miracidia arising from them. Although the integration of proviral forms and transgenes into the genome of schistosomes has been achieved (Kines *et al*. 2008; Rinaldi *et al*. 2011; Table 1), the heredity of such integrated transgenes has not yet been demonstrated. Since it is possible to transduce eggs and to obtain viable miracidia carrying the transgenes, it seems feasible to obtain germ line integration provided that the virions integrate into chromosomes of germ cells. Detecting transgenes in subsequent life cycle stages and subsequent generations would provide strong evidence for stable transformation and the general applicability of this approach.

RNAi: ELUCIDATING SCHISTOSOME GENE FUNCTION BY SMALL INTERFERING RNAs

Since classical genetic approaches analyzing gene function are not feasible in schistosomes, RNAi has become a powerful tool for functional gene analysis in this parasite (reviewed in Bhardwaj *et al*. 2011 and Hagen *et al*. 2011). Since the first reports of the successful application of this post transcriptional gene-silencing technique in *S. mansoni* (Boyle *et al*. 2003; Skelly *et al*. 2003) many studies have been published (Table 2) using this method to explore the function of genes with hypothesized roles in physiology (Cheng *et al*. 2005; Correnti *et al*. 2005; Delcroix *et al*. 2006; Krautz-Peterson and Skelly, 2008*a*; Morales *et al*. 2008; Mourão *et al*. 2009*a*, *b*; Krautz-Peterson *et al*. 2010*b*; Kumagai *et al*. 2009; McVeigh *et al*. 2011), development (Dinguirard and Yoshino, 2006; Freitas *et al*. 2007; Pereira *et al*. 2008; Rinaldi *et al*. 2009; Beckmann *et al*. 2010; Taft and Yoshino, 2011; Zou *et al*. 2011), and other aspects of biology (Tran *et al*. 2010; Yoshino *et al*. 2010; Wu *et al*. 2011). For some genes, like the schistosome thioredoxin glutathione reductase (TGR) or cAMP-dependent protein kinase (PKA), RNAi induced

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lethal phenotypes were observed by simple soaking of schistosomula (Kuntz *et al*. 2007) or electroporation of adults (Swierczewski and Davies, 2009) with corresponding dsRNAs indicating a role of these genes in worm survival. Although much was invested to improve the protocols for RNAi in adult and larval schistosomes (Krautz-Peterson *et al*. 2007, 2010*a*; Ndegwa *et al*. 2007; Stefanić *et al*. 2010), there are still some limitations. First, not all schistosomes genes can be silenced by RNAi to the same extent. Second, some genes seem to totally resist silencing and are not "knockable" at all (Krautz-Peterson *et al*. 2010*a*; Beckmann *et al*., unpublished observations). This finding is thought not to depend on the expression of the genes in specific cells that can resist RNAi, but rather to be caused by secondary structures of the target mRNAs. The latter may lead to an inaccessibility of the RNAi machinery to these folded transcripts (Krautz-Peterson *et al*. 2010*a*). Furthermore, in each RNAi experiment a number of adult or larval schistosomes was exposed to dsRNA or siRNA. Not all parasites may take up the same amount of dsRNAs/siRNAs resulting in differences in RNAi pathway activation, and thus in a high variability of gene silencing among individuals but also in different experiments. By simple soaking dsRNA or siRNA can be easily delivered to larval schistosomes (schistosomula). However, additional electroporation seems to deliver dsRNA/siRNA more efficiently into the parasites increasing the efficiency of silencing. In the case of adults (single worms or pairs), it seems that electroporation is more important to achieve a higher level of gene suppression compared to larval stages (Krautz-Peterson *et al*. 2010*a*). Operational parameters such as taking into *in vitro* culture may have an additional influence on the results of RNAi approaches since the culture media alone has a baseline effect on, for example, vitality and viability of schistosomula (Stefanić *et al*. 2010). Off-target effects, time- and dose-dependency as well as dosing limits seem to be additional critical factors for RNAi experiments in schistosomes (Stefanić *et al*. 2010). Furthermore, there is no correlation between the degree of silencing and the appearance of a phenotype. Depending on dosage effects, moderate silencing levels can also produce clear phenotypes (Freitas *et al*. 2007; Beckmann *et al*. 2010; Krautz-Peterson *et al*. 2010*a*). In contrast, a significant down-regulation of gene activity is not necessarily associated with an observable phenotype (e.g. Atkinson *et al*. 2010; McVeigh *et al*. 2011; Beckmann *et al*., unpublished observations).

Another question that remains unanswered is how dsRNA or siRNA enter larval or adult schistosomes. For newly transformed schistosomula, simple soaking alone seems to be sufficient for dsRNA delivery. Štefanić and colleagues (2010) investigated whether the gut may serve as a route for dsRNA entry into this larval stage. Using 30 μg/ml Cy5-labeled dsRNA the authors demonstrated that the gut of schistosomula takes up and concentrates the dsRNA within minutes after mechanical transformation of cercariae. Accumulation of the dye was evident along the gut and in the two terminal caecal chambers by 90 min post-transformation, and the signal remained visible during an incubation period of 6 days (Stefanić *et al*. 2010). To follow the soaking route of dsRNAs into adult schistosomes, we performed some preliminary experiments in our laboratory and incubated male schistosomes with rhodamin-labeled dsRNAs *in vitro*. The 5-carboxy-X-rhodamin was covalently linked to the dsRNA using the Label IT® Nucleic Acid Labeling Kit (Mirus Bio; USA). In each experiment, 10 males were cultivated in medium containing 5 μg labeled dsRNA/ml for up to five days. As a control, males were incubated with unlabeled dsRNA. After washing of the males, fluorescence signals were detected with a confocal laser scanning microscope (Leica TCS NT; Heidelberg) with an extinction of 597 nm. After only 2 hours incubation with rhodamin-labeled dsRNAs, weak fluorescence was detected within the excretory tubules and the flame cells (Fig. 2 B). Signal intensity increased with the time of incubation (Fig. 2 C). At day 5, fluorescence was also detected in the parenchyma (Fig. 2 D) and was no longer restricted to the excretory system. This fluorescence pattern in adult schistosomes is congruent with the staining pattern of Texas Red, which specifically stains the excretory tubules (Tan *et al*. 2003; Wippersteg *et al*. 2003). The excretory system of schistosomes has long been thought of as a route of removal for waste products. However, evidence for further functions including endocytosis was also obtained (Kusel *et al*. 2009). Our results suggest that the excretory tubules may also be involved in the uptake of dsRNAs into adult schistosomes.

Once the dsRNA is taken up *via* the gut or the excretory system of schistosomes, it has to be distributed to other tissues and cells to induce effects. In *C. elegans*, the multispan transmembrane protein SID-1 (systemic RNAi defective-1) is required for the uptake and transport of dsRNA (Feinberg and Hunter, 2003). Krautz-Peterson and colleagues recently identified *in silico* the schistosome homologue SmSID-1 (Krautz-Peterson *et al*. 2010*a*). The authors assume that SmSID-1 might also act as a channel to import dsRNA into schistosomes. Furthermore, since most schistosome tissues are syncytial, the authors speculated that once dsRNA has entered a tissue, it may be able to traverse relatively large distances without the need to cross additional membranes (Krautz-Peterson *et al*. 2010*a*). Once the dsRNA is taken up by cells, it has to be processed by the RNAi machinery to silence gene function. Up to now, a number of proteins has been identified in schistosomes representing homologues of proteins involved in the RNAi pathway of other organisms,

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Table 2. RNAi approaches in schistosomes (*S. mansoni, S. japonicum*)

Delivery method	Reference	Life cycle stage	Genetic material	Targeted schistosome gene	Silencing effect/phenotype
soaking	Boyle and Yoshino, 2003	sporocysts	dsRNA	SGTP1 (glucose transporter), GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	decreased transcript levels of the target genes; decreased glucose uptake in case of SGTP1 RNAi
	Cheng *et al*. 2005	schistosomula	siRNA	GCP (gynaecophoral canal protein)	decreased transcript and protein level
	Delcroix *et al*. 2006	3-week-old worms	dsRNA	cathepsins B1.1, L1, and D; asparaginyl endopeptidase	decreased transcript levels
	Dinguirard and Yoshino, 2006	miracidia	dsRNA	SRB (class B scavenger receptor)	decreased transcript level and sporocysts' length
	Freitas *et al*. 2007	adult pairs	dsRNA	InAct (*Inhibin*/*Activin*)	decreased transcript level; failed egg development
	Kuntz *et al*. 2007	schistosomula	dsRNA	TGR (thioredoxin glutathione reductase)	decreased TGR activity; decreased parasite movement and survival
	Nabhan *et al*. 2007	schistosomula	siRNA	RPN11/POH1 (proteasome subunit)	decreased transcript level and parasite viability
	Mourão *et al*. 2009*a*	sporocysts	dsRNA	superoxide dismutase, Smad1, Smad2, Smad4, RHO1, RHO2, Cav2A, ring box, GST26, calcineurin B, lactate dehydrogenase, EF1alpha, myosin, PKCB, HEXBP, calcium channel, Sma2, PKC receptor, DHHC, PepcK, TPx1, TPx2, calreticulin, calpain, Smeg, 14.3.3, K5, SPO1, SmZF1, GPx, fibrillarin, GST28	decreased gene expression levels was highly dependent on the selected gene, the used specific dsRNA sequence, and the timing of evaluation after treatment
	Mourão *et al*. 2009*b*	sporocysts	dsRNA	GST26 and 28, Prx1/2, GPx, SOD	decreased transcript levels; higher susceptibility to oxidative stress
	Kumagai *et al*. 2009	schistosomula	dsRNA	Prx-1/2 (peroxiredoxin-1/2)	decreased transcript level; higher susceptibility to hydrogen peroxide
	Rinaldi *et al*. 2009	eggs	dsRNA	LAP1/2 (leucine aminopeptidase 1/2)	decreased transcript and protein levels; inhibition of miracidial hatching
	Tran *et al*. 2010	schistosomula, adults	dsRNA	TSP-1/2 (tetraspanin 1/2)	changes in the tegument structure; reduced worm burden *in vivo*
	Taft and Yoshino, 2011	miracidia	dsRNA	CaM1/2 (calmodulin 1/2)	decreased transcript level; reduced growth
	Zou *et al*. 2011	schistosomula	siRNA	CRHSP-24 (calcium-regulated heat-stable protein of 24 kDa)	decreased transcript level; affected morphology and vitality
soaking, lipofection	Skelly *et al*. 2003	schistosomula	dsRNA	cathepsin B	decrease in enzyme activity

electroporation	Correnti *et al*. 2005	schistosomula	dsRNA	cathepsin B1	decreased transcript level and enzyme activity; growth retardation
	Ndegwa *et al*. 2007	adult males or females, schistosomula	dsRNA, siRNA	AP (alkaline phosphatases)	decreased transcript level and enzyme activity
	Krautz-Peterson and Skelly, 2008*a*	adult males	siRNA	AE (asparaginyl endopeptidase)	decreased transcript and protein levels
	Morales *et al*. 2008	schistosomula	dsRNA	cathepsin D	decreased transcript level and enzyme activity; significant growth retardation; dsRNA treated schistosomula did not survive to maturity after transfer into mice
	Zhao *et al*. 2008	schistosomula	shRNA expressing vector	Mago nashi	decreased transcript and protein level; changes in testicular lobes after re-introduction of the parasites into hosts
	Swierczewski and Davies, 2009	adults	dsRNA	PKA-C (cAMP-dependent protein kinase catalytic subunit)	decreased transcript and protein level; increased parasite death
	Atkinson *et al*. 2010	schistosomula	dsRNA	PAL (peptidylglycine alpha-amidating lyase)	variable and inconsistent silencing effect
	Beckmann *et al*. 2010	adult pairs	dsRNA	TK4 (Syk tyrosine kinase)	decreased transcript level; disordered oogenesis and spermatogenesis
	Krautz-Peterson *et al*. 2010*a*	schistosomula, adult pairs	siRNA	AQP (aquaporin) AP (alkaline phosphatases)	decreased transcript levels; not all schistosome genes can be suppressed to the same extent; variation in the level of suppression for one target gene
	Krautz-Peterson *et al*. 2010*b*	schistosomula adults	dsRNA siRNA dsRNA	SPRM1hc (amino acid permease heavy chain) SGTP1/4 (glucose transporter 1/4)	decreased transcript levels; impaired ability to import glucose; decreased viability *in vivo*
	Ayuk *et al*. 2011	schistosomula	shRNA-expressing vector, siRNA, dsRNA	exogeneous luciferase (luciferase was introduced as mRNA)	reduced enzyme activity
	McVeigh *et al*. 2011	schistosomula	dsRNA	npp-1 (neuropentapeptide)	decreased transcript level
	Wu *et al*. 2011	schistosomula	siRNA	tetraspanins tsp-1, tsp-3, tsp-6, tsp-8, tsp-9, tsp-12, tsp-13, tsp-14, tsp-15, tsp-17, tsp-20, Sj23, tsp-26, and tsp-76D	decreased transcript level
soaking, electroporation	Stefanić *et al*. 2010	schistosomula	dsRNA	cathepsins B1 (CB1.1, CB1.2), C, CB2, and D; annexin, Sm29, GSK-3 (glycogen synthase kinase-3), MetAP (methionine aminopeptidase), PP-2a (protein phosphatase-2a), NEC (neuroendocrine convertase), nMT (n-myristoyl transferase)	decreased transcript levels; selective transcript suppression; variant sensitivity of suppression

Table 2. (*Cont.*)

Delivery method	Reference	Life cycle stage	Genetic material	Targeted schistosome gene	Silencing effect/phenotype
electroporation, soaking, lipofection	Krautz-Peterson *et al*. 2007	schistosomula	dsRNA	cathepsin B1	decreased transcript level and enzyme activity
particle bombardment	Osman *et al*. 2006	adults	siRNA	TGFβ-RII (transforming growth factor-beta receptor II)	decreased transcript level of SmTβ-RII and reduced expression of SmGCP
retroviral transduction	Tchoubrieva *et al*. 2010	adults	dsRNA hairpin-expressing viral vector	cathepsin B1	decreased transcript level and enzyme activity
injection into infected mice	Pereira *et al*. 2008	adults *in vivo*	siRNA	HGPRTase (hypoxanthine-guanine phosphoribosyltransferase)	reduction in parasite target mRNA but not of the homologous host target; reduced number of parasites
soaking, injection into infected mice	Cheng *et al*. 2009	12-day-old worms, adults *in vivo*	siRNA	GCP (gynaecophoral canal protein)	decreased transcript and protein levels; abolition of pairing *in vitro* and *in vivo*; reduced worm burden *in vivo*

[dsRNA: double stranded RNA; siRNA: small interfering RNA; shRNA: small hairpin-RNA].

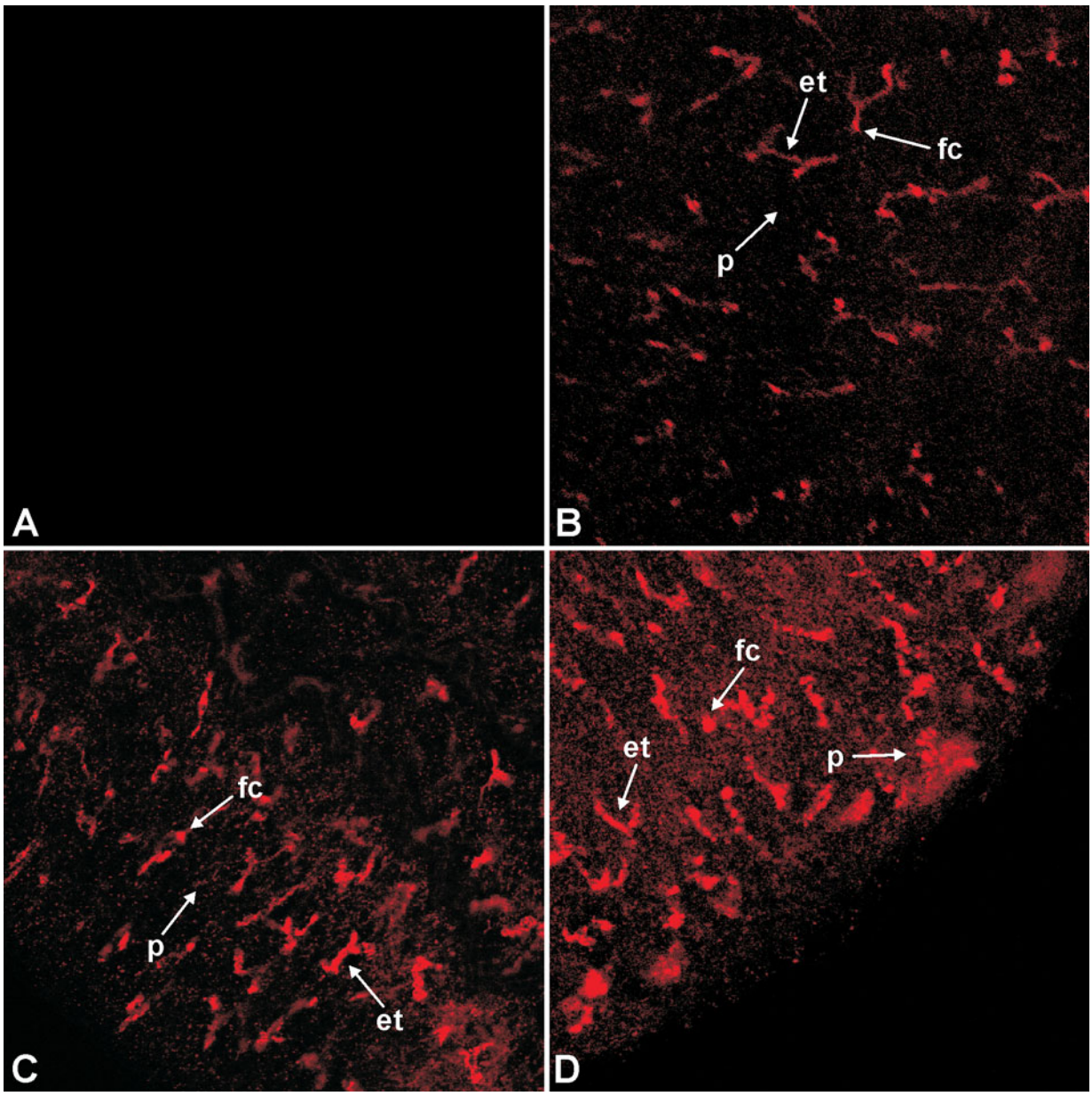

Fig. 2. Adult *S. mansoni* males following *in vitro* incubation with rhodamin-labeled dsRNAs after 2 hours (B), 3 days (C), and 5 days (D), or as control with unlabeled dsRNA (A). Fluorescence signals were detected by confocal laser scanning microscopy (Leica TCS NT) with an extinction of 597 nm. [et: excretory tubules, fc: flame cells, p: parenchyma].

which indicates a similar scenario in schistosomes (Krautz-Peterson *et al*. 2010*a*).

Vector-based dsRNA and siRNA/shRNA delivery

Vector-based approaches have been successfully used to deliver small RNAs (dsRNAs, siRNAs) in gene silencing experiments. Zhao and colleagues (2008) first reported vector-mediated gene silencing in *S. japonicum*. They used siRNAs delivered from short hairpin-RNAs (shRNAs) expressed *in vivo* in schistosomules by a mammalian Pol III promoter H1 and successfully suppressed a Mago nashi gene (Zhao *et al*. 2008). Similarly to this approach Ayuk *et al*. (2011) established a vector-based RNAi technique for *S. mansoni* using a plasmid expressing shRNAs driven by the schistosome U6 gene promoter. They demonstrated that a shRNA targeting reporter firefly luciferase reduced firefly luciferase mRNA and luciferase enzymatic activity in transformed schistosomules (Ayuk *et al*. 2011). Tchoubrieva and colleagues (2010) designed a viral vector expressing a dsRNA hairpin to silence the expression of the schistosome cathepsin B1 (SmCB1) gene and were the first to show that this approach also works in adult schistosomes. The vector-based delivery of dsRNAs or shRNAs could be a preferable technique for the

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investigation of long-term silencing effects of specific mRNAs compared to the transient suppression of gene expression achieved by soaking or electroporation.

Large-scale RNAi approaches

De Moraes Mourao and colleagues (2009*a*) made the first attempt to use RNAi for large-scale screening approaches. They selected 32 different genes and analyzed potential morphological changes in sporocysts after application of corresponding dsRNAs. Their results indicated that the efficiency of altered gene expression due to dsRNA-treatment is highly variable and dependent on (1) the selected target gene, (2) the selected dsRNA sequence within the target gene, and (3) the timing of evaluation after treatment. The authors concluded that potential off-target effects, non-specific effects of some dsRNAs, and variable efficiencies of specific gene silencing still are critical points of RNAi in schistosomes (de Moraes Mourao *et al*. 2009*a*). Optimization is needed as well as careful gene-specific testing as part of RNAi experiments and data interpretation. In addition, improvements of dsRNA delivery methods were discussed as being critical. Regarding large-scale RNAi experiments, Mourão and colleagues concluded that low and inconsistent dsRNA uptake, the low number of parasites that can be processed in a single treatment, the limited phenotype repertoire, and the lack of more sensitive detection tools currently restrict large-scale approaches. Thus RNAi seems presently to be suitable only for small scale or gene-by-gene characterization approaches (Mourão *et al*. 2009*a*). Stefanić and colleagues (2010) tried to define operational parameters, which may facilitate larger RNAi screening and suggested the use of newly transformed schistosomula due to handling advantages.

In vivo *RNAi approaches*

A few approaches have been undertaken using siRNAs as therapeutical agents. Pereira and colleagues (2008) successfully used *in vivo* RNAi to reduce worm burdens in mice chronically infected with *S. mansoni*. SiRNAs targeting the hypoxanthine-guanine phosphoribosyl-transferase (HGPRTase) were intravenously injected in infected mice. This led to a significant reduction of the total number of parasites after six days as well as to a reduction of the parasite target mRNA, but not of its host's homologue (Pereira *et al*. 2008). Cheng *et al*. (2009) used a similar approach to knock down the gynaecophoral canal protein of *S. japonicum* (SjGCP) *in vivo*, which led to a reduction in parasite pairing and total worm burden. These results indicate that *in vivo* RNAi may also be possible.

UNANSWERED QUESTIONS ON THE WAY TO TRANSGENIC SCHISTOSOMES

Over the last decade, much effort and enthusiasm have been invested into the establishment of systems to generate transgenic schistosomes. Different innovative approaches were performed indicating their suitability for transient transformation/transfection of this multicellular parasite. Although the first transgene integrations into schistosome chromosomes have been achieved, there is still a long way to go towards obtaining stably transformed schistosomes.

Are miracidia and eggs the only life cycle stages useful as targets for transformation approaches with the aim to enter the germ line? Both appear to be preferable stages, because their germ cells seem to be easily accessible using particle bombardment or retroviruses. Furthermore, miracidia, either directly transformed, transfected or hatching from transfected eggs, can be reintroduced into the schistosome life cycle by snail infection. In theory, although they differ in the number of germ cells, sporocysts, schistosomula or adults also represent potential targets, but they must be reintroduced into the life cycle by implanting into intermediate or final hosts, respectively. The implantation of sporocysts into snails (Jourdane and Theron, 1980; Jourdane *et al*. 1985) or of schistosomula and adults into final hosts (Basch and Humbert, 1981) is possible, but the approaches are technically demanding and time consuming.

How can we achieve a tissue-specific knock-down of gene activity? If stable transformation of schistosomes could be achieved, the door would open for a number of fundamental analyses in this direction. For example, by integrating a transgene cassette expressing a dsRNA or shRNA under the control of certain schistosome gene promoters, tissue-specific RNAi experiments would be possible in various different life stages. Problems with tissue-dependent off-target effects as well as with the dsRNA/shRNA delivery and its intra-organism transport could be overcome. Concerning this point, it is still not absolutely clear how dsRNAs, shRNAs or siRNAs enter the parasite finding their way to different tissues and cells, and whether all tissues and cells can be reached. Especially for adults we cannot exclude that not all tissues are accessible to dsRNAs, shRNAs or siRNAs to the same degree and that in different tissues RNAi effects might be effective to different extents due to variable concentrations of these nucleic acids or other factors.

Are all members of the RNAi machinery expressed to the same degree in all tissues of schistosomes, and in all life stages? With respect to the observation that some genes can be effectively knocked down in contrast to others may hint at the possibility that 'non-knockable' genes are expressed in tissues (1) which cannot be reached by dsRNAs/shRNAs/siRNAs, or (2) in which not all components of the RNAi

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processing pathway are present. Localization experiments employing whole mount *in situ* hybridization recently showed that transcription of the *S. mansoni* Argonaute 2 (Smago2), an essential member of the RNAi pathways (Ketting, 2011), is restricted to the ovary, the vitelline glands and testes of adult worms (Cogswell *et al*. 2011). Since the used hybridization probe was specific for Smago2, it cannot be excluded that the *S. mansoni* homologues of ago1 and/or ago3 transcripts were present in other tissues fulfilling ago2-like, redundant RNAi functions. Thus, further localization experiments are needed to ascertain the expression patterns of relevant members of the predicted schistosome RNAi pathway. Using RT-PCR analyses, the expressions of the schistosome Dicer homologues (SmDicer, SjDicer) and schistosome Argonaute homologues (SjAGO1, 2, 3) were shown in different life cycle stages with the highest expression in larval stages (Krautz-Peterson and Skelly, 2008*b*; Luo *et al*. 2010) demonstrating that, in principle, all life stages might be susceptible to RNAi, but also indicating that RNAi could be more effective in the larval stages. However, it still has to be elucidated why not all schistosome genes seem to be susceptible to RNAi, and whether factors like the localization or conformation of the target mRNA or as yet unknown factors may additionally influence RNAi in schistosomes.

Stably transformed schistosomes seem to be the most attractive solution to problems with delivery, uptake and variable concentrations of ds/sh/siRNAs in different tissues. Provided that gene cassettes expressing such RNAs are not silenced at the genomic level by epigenetic factors such as methylation post transformation, as observed in plant transgenesis (Matzke *et al*. 2000; Fischer *et al*. 2006), such integrated transgenes would ensure the constant delivery of RNAs and probably continuous silencing effects.

Summarizing we would like to make the point that combining the techniques already available today might be the way to reach this goal in the near future. For example, with a combination of transposon or retrovirus-based systems and particle bombardment of miracidia it should not only be possible to reach the germ cells and to achieve the integration of transgenes into their genomes, but also to reintroduce transgenes *via* miracidia into the schistosome life cycle and to monitor their heredity during life cycling through subsequent generations.

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