Published in final edited form as:
Parasitology. 2012 April ; 139(5): 641–650. doi:10.1017/S003118201100134X.

Genetic manipulation of schistosomes – progress with integration competent vectors

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SUMMARY

Draft genome sequences for *Schistosoma japonicum* and *S. mansoni* are now available. The schistosome genome encodes ~13000 protein-encoding genes for which the functions of few are well understood. Nonetheless, the new genes represent potential intervention targets, and molecular tools are being developed to determine their importance. Over the past 15 years, noteworthy progress has been achieved towards development of tools for gene manipulation and transgenesis of schistosomes. A brief history of genetic manipulation is presented, along with a review of the field with emphasis on reports of integration of transgenes into schistosome chromosomes.

Keywords

Schistosomes; genetic manipulation; transgenesis; chromosome integration; germ line; retrovirus; murine leukaemia virus; pseudotyped gammaretrovirus; transposon; *piggyBac*

INTRODUCTION

Schistosomes are considered the most important of the human helminth infections in terms of morbidity and mortality. More than 200 million people are infected with schistosomes and a further 800 million are at risk of schistosomiasis in >75 countries in tropical and sub-tropical latitudes. Treatment and control of schistosomiasis rely on the anthelmintic drug praziquantel, but there is concern that drug resistance will appear. New interventions, including vaccines, drugs and diagnostics, are needed for this neglected tropical disease (Hotez *et al.* 2008; Brindley *et al.* 2009, and references therein).

Draft genome sequences for *Schistosoma japonicum* and *S. mansoni* were reported recently, a landmark event that ushered in the post-genomic era for schistosomiasis (*Schistosoma japonicum* Genome & Functional Analysis Consortium, 2009; Berriman *et al.* 2009; Han *et al.* 2009). Despite the abundant new datasets, functional analysis of target genes to underpin new interventions for schistosomiasis requires routine approaches for both reverse and forward genetics. To date, functional genomics approaches beyond conventional RNA interference have not been available for schistosomes although reporter plasmids and RNAs

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have been introduced to several developmental stages (e.g. Davis *et al.* 1999; Wippersteg *et al.* 2002*b*, 2005; Mourão *et al.* 2009; Krautz-Peterson *et al.* 2010; Stefanic *et al.* 2010). Functional genomics including somatic and germline transgenesis are desirable because these techniques facilitate validation of essential genes/gene products to be targeted with drugs or vaccines (e.g. van Ooij *et al.* 2008; Homann *et al.* 2009; Langridge *et al.* 2009; Buguliskis *et al.* 2010). This review addresses genetic manipulation of schistosomes. More specifically, this review focuses on reports of genetic manipulation of schistosomes dealing with approaches targeting integration of transgenes into schistosome chromosomes.

BRIEF HISTORY OF GENETIC MANIPULATION IN SCHISTOSOMES

Advances with genetic manipulation of parasitic helminths including schistosomes have been reviewed (Grevelding, 2006; Brindley and Pearce, 2007; Kalinna and Brindley, 2007; Alrefaei *et al.* 2011; Mann *et al.* 2011). In brief, transgenesis of schistosomes was pioneered by Davis and co-workers who bombarded adult stages of *S. mansoni* with mRNA-encoding firefly luciferase and a luciferase-encoding plasmid (Davis *et al.* 1999). Subsequently, Grevelding and colleagues undertook a series of studies employing particle bombardment of *S. mansoni* stages using plasmids co-precipitated on gold beads. The plasmids encoded fluorescent reporter proteins and were driven by promiscuous (e.g. HSP70) or tissue-specific gene promoters (e.g. cathepsin F) from schistosomes (Wippersteg *et al.* 2002*a*, b, 2003, 2005; Rossi *et al.* 2003; Dvorak *et al.* 2010).

Heyers and colleagues introduced plasmid DNA (coated on gold beads) into miracidia, sporocysts and adults of *S. mansoni* by particle bombardment (Heyers *et al.* 2003). The plasmid construct encoded green fluorescent protein (GFP) under control of the *S. mansoni* HSP70 (heat shock protein 70 kDa) promoter and termination elements (Wippersteg *et al.* 2002*b*). The bombarded larvae and adults expressed GFP, and the transformed miracidia penetrated and established in the intermediate snail host *Biomphalaria glabrata*. Gold particles were detected in the germ balls of parasites in snail tissue, indicating feasibility of returning transformed parasites to the developmental cycle, a step expected to be useful for establishing lines of transgenic schistosomes.

Correnti and Pearce (2004) demonstrated that square wave electroporation could introduce reporter genes into schistosomes. Subsequently, the technique has found wide acceptance for introduction of plasmids, long dsRNA, siRNA, virions and other reporters into all three major species of human schistosomes, *S. mansoni* (e.g. Morales *et al.* 2008; Dvorak *et al.* 2010), *S. japonicum* (Zhao *et al.* 2008) and *S. haematobium* (Rinaldi, unpublished). Square wave electroporation has been successfully employed to introduce nucleic acids into eggs, miracidia, sporocysts, schistosomules and adult developmental stages of schistosomes, frequently using a single pulse of 125 volts for 20 milliseconds, in 4 mm gap pathway cuvettes (e.g. Correnti *et al.* 2005; Faghiri and Skelly, 2009; Kines *et al.* 2010). Moreover, electroporation has also been employed to develop a method to assess for the presence of an active RNAi pathway by silencing the exogenous reporter gene, firefly luciferase. This straightforward approach offers investigators a means to study the presence of a functional RNAi pathway in less well known parasites and/or to detect the activity of non-conventional interfering molecules such as short hairpin RNAs in schistosomes (Rinaldi *et al.* 2008; Ayuk *et al.* 2011).

Findings reported by Grevelding and colleagues are notable in relation to the introduction of transgenes into the germline of schistosomes (Grevelding, 2006; Beckmann *et al.* 2007). Plasmids (both super-coiled and linear) encoding GFP were introduced into miracidia by particle bombardment, after which the transformed miracidia infected *Biomphalaria glabrata* snails by the natural route. Investigation of the cercariae (F0 generation) shed from the snails

and adult worms from hamsters infected with the cercariae and of eggs (F1) from the rodents revealed the presence of the GFP transgene. Miracidia harvested from eggs obtained from the livers of the hamsters were used for snail infections and the resulting cercariae were employed to infect hamsters to derive subsequent schistosome generations, F2 and F3. Molecular analyses of F2 and F3 cercariae or adults failed to detect transgenes. Nonetheless, the findings demonstrated that the transgenes were passed from one developmental 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 invariable cell type in the developmental cycle of the schistosome, the investigators concluded that the transgenes were present in the germline and their germline-transformation approach had succeeded. Loss or instability of the transgene before (non-Mendelian) inheritance by the F2 progeny likely occurred because transgenes had not integrated into the schistosome genome, a phenomenon well known with extrachromosomal arrays of transgenes in *Caenorhabditis elegans* (see Semple *et al.* 2010). Fig. 1 provides a time line - over the past 15 years - of the pioneering and key advances in the genetic manipulation of schistosomes.

SCHISTOSOME TRANSGENESIS WITH INTEGRATION COMPETENT VECTORS

Although approaches to genetic manipulation of schistosomes with non-integration competent vectors have been informative, there are major advantages to genomic integration of transgenes including Mendelian inheritance, sustained transgene activity and transgene-vectored RNA interference (see Giordano-Santini and Dupuy, 2011). Several classes of integration competent vectors enjoy utility in functional genomics and gene therapy for a spectrum of eukaryotes. These include transposons, gammaretroviruses, lentiviruses and recombinase systems (e.g. see Mates *et al.* 2007; Damasceno *et al.* 2010; Turan *et al.* 2011). Several of these are now being actively investigated for utility in integrating transgenes into the schistosome genome (Alrefaei *et al.* 2011).

Retroviruses

Both simple and complex retroviruses (family Retroviridae) are widely employed in functional genomics and gene therapy biotechnologies (e.g. see Hannon and Rossi, 2004; Petrus *et al.* 2010; Sliva and Schnierle, 2010). The simplex retroviruses include the genus *Gammaretrovirus* which includes the murine leukaemia virus (MLV). Complex retroviruses include the genus *Lentivirus* which includes the primate pathogens, HIV and SIV. Attributes of retroviruses that advance their appeal as gene transfer vectors include self-reliant infectious nature, ability to integrate into the chromosomes of the infected cell, potential to be modified to increase host cell range and availability of numerous constructs from commercial sources and academic colleagues.

For safety, retroviral vector systems are usually employed in two components – the retroviral vector, which does not encode viral proteins and the retrovirus packaging cell line, which provides the viral proteins necessary for vector transfer. Infectious, but replication incompetent virions are released from transfected packaging cells. The virus can infect target cells but cannot produce new virions after integration into host chromosome because the integrated provirus does not encode viral proteins (Miller, 1992). The restricted host-cell range of retroviral vectors limits their use for stable gene transfer in eukaryotic cells. To overcome this latter limitation, Burns and colleagues pioneered to use of vesicular stomatitis virus glycoprotein (VSVG) pseudotyped murine leukaemia virus (MLV)-derived vectors in which the retroviral envelope glycoprotein is replaced by the glycoprotein (G) of the rhabdovirus, vesicular stomatitis virus (VSV) (Burns *et al.* 1993). VSVG is able to bind to phospholipids on membranes of eukaryotic cells at large, endowing the VSVG pseudotyped

virion with a potentially very broad range of target tissues and cells (Mastromarino *et al.* 1987; Emi *et al.* 1991). VSVG pseudotyped virions can infect non-mammalian cells including fish cell lines that are ordinarily refractory to infection because they do not express a cognate receptor for the envelope (surface) protein ligands of the wild type virions.

In our laboratory it has been well established that the infectious replication incompetent MLV retrovirus pseudotyped with VSVG can transduce *S. mansoni* leading to integration of retroviral transgenes into schistosome chromosomes (Kines *et al.* 2006, 2008, 2010; Rinaldi *et al.* 2011). This was notable given it was thought that evolutionary blocks would constrain the utility of MLV in non-mammalian taxa (Dirks and Miller, 2001). The MLV-derived vector pLNHX was modified to include reporter genes (firefly luciferase or green fluorescent protein) under the control of an endogenous schistosome gene promoter – the RNA polymerase II (Pol II) schistosome actin gene promoter or the RNA polymerase III (Pol III) Spliced Leader (SL) RNA gene promoter. Constructs and the plasmid encoding VSVG were employed to transfect GP2-293 packaging cells modified to express the MLV *gag* and *pol* genes (Mann *et al.* 2008). Eggs, primary sporocysts, schistosomules and adult stages of *S. mansoni* have been successfully transduced with the VSVG pseudotyped MLV virions. Two-colour immunofluorescence, Southern hybridization and RT-PCR confirmed successful transduction of the schistosomes by this gammaretrovirus. Furthermore, an anchored PCR (retrotransposon-anchored PCR, RAP) approach that employs primers specific for multi-copy endogenous mobile genetic elements interspersed in the schistosome genome was successfully deployed to locate integration junctions of transgenes in the genome of *S. mansoni*, definitively establishing the presence of proviral MLV transgenes integrated into schistosome chromosomes (Kines *et al.* 2008). In terms of promoters, schistosome actin, HSP70 and spliced leader (SL) gene promoters, as well as the 5′-LTR of MLV, all were found to drive transgene expression in viriontransduced schistosomes (Kines *et al.* 2006, 2008).

It is likely that the schistosome cells transduced by the virions were frequently tegumental and/or intestinal cells (Mann *et al.* 2008). However, we have also fragmented adult worms into several pieces before exposing the (still visibly motile) fragments to virions, which resulted in increased density of transgenes integrated into the schistosome chromosomes (Rinaldi *et al.* 2011). Nonetheless, in order for heritable transmission to occur, germline transduction would have to have taken place. The schistosome egg represents an advantageous developmental stage at which to direct transgenes (Kines *et al.* 2010; Mann *et al.* 2011). Accordingly, we proceeded to transduce schistosome eggs with VSVG-pseudotyped MLV facilitated by electroporation. Square wave electroporation was more effective in delivering VSVG-pseudotyped MLV into schistosome eggs than passive soaking. Quantitative PCR (qPCR) analysis revealed that schistosome eggs electroporated with virions had several fold more copies of provirus than eggs simply soaked in virions (Kines *et al.* 2010). These findings highlight the potential of the schistosome egg as a target into which to deliver chromosomal integration competent transgenes, aiming to establish germline transgenesis in schistosomes.

VSVG-pseudotyped MLV has been employed to transfer transgenes into *S. japonicum*; Yang *et al.* (2010) transduced schistosomules (perfused from rabbits) with retroviral transgene encoding human telomerase reverse transcriptase (hTERT). RT-PCR, *in situ* hybridization immunohistochemistry and immunoblot analysis determined that *S. japonicum* can be successfully transduced with VSVG-pseudotyped MLV and that the MLV vector can transport sizeable genes as cargo – the hTERT gene was ~3.5 kb in length (Yang *et al.* 2010). These findings also suggested the tantalizing possibility of using the hTERT transgene to immortalize cells from schistosome tissues, utilizing the oncogenic potential of hTERT to establish schistosome cell lines.

Transposons

Transposons are naturally occurring mobile genetic elements that move by a cut-and-paste mechanism; they are flanked by inverted terminal repeat (ITR) sequences and mobilized by a transposase encoded by their single open reading frame. There are ~20 superfamilies of these Class II mobile genetic elements, with member species widespread throughout eukaryote phyla (Feschotte and Pritham, 2007; Yuan and Wessler, 2011). Several are known from schistosomes including examples of the *Merlin* and CACTA groups (Berriman *et al*. 2009). Transposons can frequently mobilize in species phylogenetically distant from where they were first isolated, a facility which has been harnessed in functional genomics and experimental gene therapy (Plasterk *et al*. 1999; Ivics *et al*. 2009). Accordingly, it is feasible that exogenous transposons might also mobilize in schistosomes and thereby supply integration competent vectors for functional genomics of schistosomes. Several well-studied transposons including *piggyBac, Hermes* and *mariner*, are transpositionally active in planarians (Gonzalez-Estevez *et al*. 2003). In evolutionary terms, it is notable that host-parasite interactions play a key role in the horizontal transfer of transposons across phyla (Gilbert *et al*. 2010).

The *piggyBac* transposon is used widely in functional genomics and experimental gene therapy (Gonzalez-Estevez *et al*. 2003; Balu *et al*. 2005; Wilson *et al*. 2007). This transposon was isolated from the genome of a moth. It is a short inverted terminal repeat element of 2.5 kb in length with ITRs of 13 bp in length and a single open reading frame encodings the transposase. *piggyBac* exhibits precise excision upon transposition and affinity for TTAA target sites (Fraser *et al*. 1985, 1996; Cary *et al*. 1989; Elick *et al*. 1996). Recently, it has been determined that *piggyBac* is also active in schistosomes. Morales and colleagues examined whether the *piggyBac* transposon could deliver reporter transgenes into the genomes of *S. mansoni* (Morales *et al*. 2007). Linearized *piggyBac* donor plasmid carrying the firefly luciferase gene as reporter cargo under the control of schistosome gene promoters – actin (pXL-BacII-SmAct-Luc) or HSP70 (pXL-BacII-SmHSP70-Luc) – was introduced together with mRNA encoding the *piggyBac* transposase into cultured schistosomules by square wave electroporation. Activity of the helper transposase mRNA was confirmed by hybridization of genomic DNA from the transformed schistosomes to a luciferase gene probe. The hybridization signals indicated that the *piggyBac* transposon had integrated into numerous sites within schistosome chromosomes. Integration events were recovered using an anchored PCR approach employing several endogenous mobile genetic elements from the schistosome genome as anchors, which revealed characteristic *piggyBac* TTAA footprints in the vicinity of several protein encoding genes, annotated as adenylosuccinate lyase, glutathione peroxidase 1 and glutathione S transferase, as well as loci near endogenous mobile genetic elements including *Boudicca* and *SR2*. These findings provided the first direct evidence of somatic transgenesis of schistosomes, or indeed of any parasitic helminth. They demonstrated the transpositional activity of *piggyBac* in schistosomal tissues, expanding the host range of *piggyBac* to the digenetic trematodes. Very recently, we reported that vector-based RNAi activity driven by transgene cargo carried by the *piggyBac* vector pXL-BacII (Ayuk *et al*. 2011) – see below.

Other integration competent vectors

We are unaware of reports of other integrated transgenes in schistosomes beyond the findings with MLV and *piggyBac* (above). However, it is not unlikely that other retrovirus and transposons, including endogenous schistosome mobile genetic elements, could find utility in genome studies of schistosomes. A potential advantage of endogenous mobile genetic elements is that they may not suffer infection blocks from host restriction/innate immunity factors (e.g. Takeuchi and Matano, 2008; Strebel *et al*. 2009). DNA site-specific recombinases (SSRs), such as Cre, FLPe and φC31, from bacteriophages of fungi, and other

provenances are influential tools for analyzing gene function in vertebrates (e.g. see Bischof and Basler, 2008) and might also be active in schistosomes. Several are now in service for site-specific gene manipulation of *Plasmodium falciparum* (Adjalley *et al*. 2010; O'Neill *et al*. 2011).

In overview, transgenesis mediated by integration competent vectors such as MLV and *piggyBac* can provide a routine functional genomics platform for forward and reverse genetics of schistosomes. Forward genetics, where specimens displaying a mutant phenotype after insertional (or chemical, etc.) mutagenesis are selected – the `from phenotype to genotype' approach – can now be attempted using transduction of schistosomes (e.g. with MLV or *piggyBac*) followed by high throughput sequence analysis of the schistosome genome in similar fashion to other pathogens (e.g. Langridge *et al*. 2009). Further, given that draft schistosome genomes are now available, MLV or *piggyBac* can be used for reverse genetics. With reverse genetics, functional analysis involves targeting a known gene sequence for inactivation where the function of the gene of interest is then inferred from the resulting phenotype (`from genotype to phenotype' approach) (Boutros and Ahringer, 2008). Conventional RNAi and more recently vector-based RNAi in schistosomes are the reverse genetic tools of choice for reverse genetics, approaches widely used for discovery of targets for experimental drug and/or vaccine development (e.g. Sayed *et al*. 2006; Mourão *et al*. 2009; Stefanic *et al*. 2010).

VECTOR-BASEDRNAi

Experimental RNAi works well in schistosomes, in general (see Krautz-Peterson *et al*. 2010). Skelly and co-workers and Boyle and colleagues first described successful knockdown in *S. mansoni* (Boyle *et al*. 2003; Skelly *et al*. 2003), and since then numerous reports describing endogenous and reporter gene knockdown in *S. mansoni* and *S. japonicum* have appeared (e.g. Kumagai *et al*. 2009; Rinaldi *et al*. 2009). RNAi is active in *S. haematobium* (Rinaldi and co-workers, unpublished). However, conventional RNAi by double stranded RNA frequently leads to transient gene silencing and, in addition, may be inaccessible to some developmental stages and/or tissues of schistosomes. *In vivo*, e.g. vector-based RNAi approaches that lead to integration of transgenes encoding cassettes that express short interfering RNAs can circumvent deficiencies with exogenous RNAi approaches by providing continuous and/or conditional gene silencing (see Sliva and Schnierle, 2010). In brief, these experimental systems frequently employ a gene construct encoding an oligonucleotide of the target siRNA, a short loop domain (~9 residues), followed by the reverse complement of the siRNA, and driven by a Pol III (or Pol II) promoter. The construct can then be introduced into target cells for endogenous expression of shRNA targeting the gene of interest. The shRNA is processed in the cytoplasm to siRNA (Manjunath and Dykxhoorn, 2010). Both plasmid-based and retroviral (integrating) vectors are widely used for vector-based RNAi procedures, the latter offering long term gene silencing of expression (Couto and High, 2010; Sliva and Schnierle, 2010). Zhao and co-workers pioneered the approach in schistosomes, demonstrating silencing of the expression of the *Mago nashi* gene in *S. japonicum* by siRNAs derived from shRNA expressed by mammalian Pol III promoter H1 (Zhao *et al*. 2008). We recently demonstrated that MLV encoding long hairpin RNAs, ~120 bp long (hpRNA), driven by a RNA polymerase II promoter (*S. mansoni* actin) targeting *S. mansoni* cathepsin B in the adult stage of *S. mansoni* delivered silencing of the protease (Fig. 2, left panel) (Tchoubrieva *et al*. 2010). On the other hand, in many species including insects, mammals, birds and pathogenic protozoa, Pol III promoter-based DNA vectors have been employed to express small interfering RNA (siRNA) or short hairpin RNA (~21 bp long) (shRNA) (Lambeth *et al*. 2005; Wakiyama *et al*. 2005; Wise *et al*. 2007; Linford *et al*. 2009). Aiming to establish vector-based RNAi driven by a Pol III promoter, we cloned *S. mansoni* and human U6 gene promoters (~270

bp) into pLNHX driving shRNA targeting firefly luciferase. We targeted luciferase because the effect of RNAi against luciferase can be readily discerned (in contrast to many endogenous genes) (Rinaldi *et al*. 2008). Luciferase activity was significantly reduced in worms transduced with *piggyBac* encoding shLuc (Fig. 2, right) (Ayuk *et al*. 2011).

DEVELOPMENTAL STAGES OF SCHISTOSOMES FOR TRANSGENESIS

Since developmental cycles of the three major schistosomes of humans can be accomplished using laboratory rodents as the mammalian hosts and laboratory-reared snails as the intermediate hosts (Lewis, 1998), most developmental stages of these schistosomes are theoretically accessible to genetic manipulation (Fig. 3). Moreover, some stages can be cultured *ex vivo* or *in vitro* and returned to the snails or mice to continue development (see Mann *et al*. 2010). On the other hand, discrete stages are differentially accessible to delivery of transgenes using approaches including particle bombardment, square wave electroporation, cationic polymer-based gene delivery, and/or transduction by virions or other infectious agents (Heyers *et al*. 2003; Beckmann *et al*. 2007; Mann *et al*. 2008). Other approaches, such as microinjection, should be of value, as indicated by progress with introduction of transgenes in tape-worms and parasitic nematodes (Spiliotis *et al*. 2010).

Adult worms can be obtained from infected rodents and can be maintaining in culture. `Viable' fragments of worms – obtained by dicing adult schistosomes into several pieces – can be used as a study model as well (Rinaldi *et al*. 2011); whereas these fragments are not as tractable as primary cell cultures of *Echinococcos multilocularis* (Spiliotis *et al*. 2010) or fragments of planarians (Shibata *et al*. 2010), they do allow access to internal organs and cells of the schistosome. The schistosome egg and the miracidium have desirable attributes for consideration in relation to genetic manipulation, these include the presence of a single celled zygote within the egg-shell upon its release from the blood fluke (Jurberg *et al*. 2009), favourably high ratio of germ to somatic cells even as it develops and ease of maintenance *in vitro*. Primary sporocysts transformed from miracidia *in vitro* are worthy targets for genomic manipulation because this developmental stage can be transplanted into *Biomphalaria glabrata* snails to establish lines of *S. mansoni* (Kapp *et al*. 2003). Schistosomules obtained by mechanical transformation of cercariae shed from snails have been used to investigate the activity of transgenes and/or schistosome gene promoters driving transgenes (Correnti *et al*. 2005, 2007; Morales *et al*. 2007).

The `*in vitro* laid egg' (IVLE) deserves special mention. Pearce and colleagues demonstrated that eggs develop after release from adult schistosomes *in vitro* (Freitas *et al*. 2007). Eggs released from the fertilized adult female schistosome can develop *in vitro* and eventually release viable miracidia, provided that the eggs have been laid soon after the adult worms have been perfused from experimentally infected rodents. (From about 48 hours after perfusion from mice, eggs shed from worms exhibit reduced viability.) Notably, when released from the female worm, the schistosome egg includes a single cell zygote, in which cleavage has yet to take place (Jurberg *et al*. 2009). Accordingly, introduction of transgenes into this young egg may be able to accomplish germ (and somatic) transgenesis in a developmental stage that seems to be reasonably accessible in the laboratory (Mann *et al*. 2011).

Finally, it is obvious that the availability of immortalized cell lines would enhance functional genomics investigations (Brindley and Pearce, 2007). Unfortunately, none are yet available. Progress with primary cell cultures in related flatworms (Spiliotis *et al*. 2010) indicates that cell lines can be established and perhaps progress with transgenesis of schistosomes with oncogenes such as hTERT (Yang *et al*. 2010) will provide a route forward in this area.

PERSPECTIVE

We have reviewed advances in functional genomics and transgenesis of schistosomes, focusing on approaches leading to chromosomal integration of transgenes. The retrovirus MLV and the transposon *piggyBac* have now both been shown to integrate reliably into the chromosomes of *S. mansoni* and hence both exhibit great potential as vectors to drive functional genomics for schistosomes. However, improvements are needed to establish transgenic schistosomes and protocols. An impediment has been the difficulty of delivering transgenes to the germline. Targeting integration competent transgenes to IVLE may surmount this roadblock (Mann *et al*. 2011). Other gateways to the schistosome germline include the daughter sporocysts where the germ cells are comparatively massive (see Coustau and Yoshino, 2000). We envisage that advances in technologies which will drive functional genomics forward quickly, including expansion of *in vivo* RNAi, high-throughput insertional mutagenesis and, hopefully, gains-of-function approaches involving drug selection of transgenic schistosomes. Advances in *S. mansoni* can be expected to be adapted to the other schistosomes, to the food-borne flukes such as *Opisthorchis viverrini, Clonorchis sinensis* and *Fasciola hepatica*, and other helminth parasites at large.

Acknowledgments

Support from NIH-NIAID award R01AI072773 is gratefully acknowledged. We thank Tunika Okatcha and Danielle Skinner for critical review of the manuscript.

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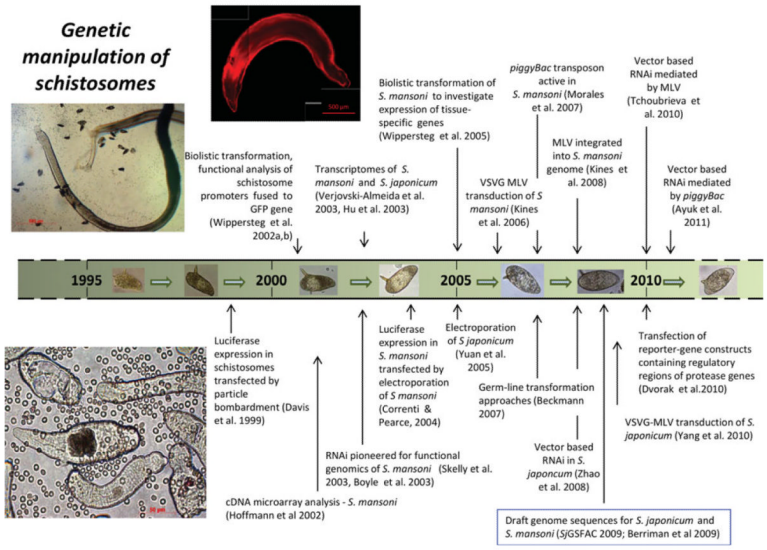

Fig. 1.

Schematic time line of advances on genetic manipulation of schistosomes. Key events are noted, with pioneering or key reports cited.

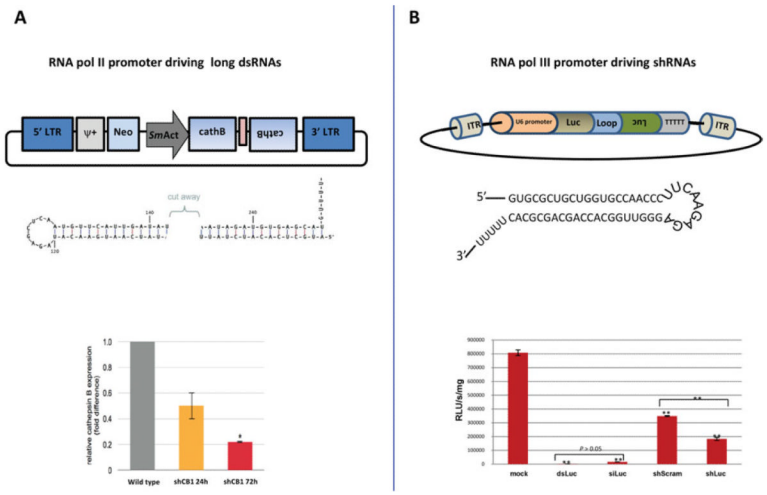

Fig. 2.

Vector-based RNA interference (RNAi) in *Schistosoma mansoni*. Panel A: Vector-based RNAi mediated by long hairpin RNAs (hpRNA) driven by a RNA polymerase II promoter (from the S. mansoni actin gene) carried by a retroviral vector to knock down of the *S. mansoni* cathepsin B1. Top: schematic representation of retroviral vector construct and dsRNA hairpin. Bottom: knock down of *S. mansoni* cathepsinB1. Panel B: Vector-based RNAi mediated by short hairpin RNAs (shRNA) driven by a RNA polymerase III promoter (from the *S. mansoni* U6 gene) carried by the *piggyBac* donor plasmid to knock down of the exogenous reporter gene firefly luciferase. Top: schematic representation of construct encoding the shRNA. Bottom: knock down of reporter firefly luciferase activity. Adapted from Tchoubrieva et al. (2010) and Ayuk et al. (2011) with permission.

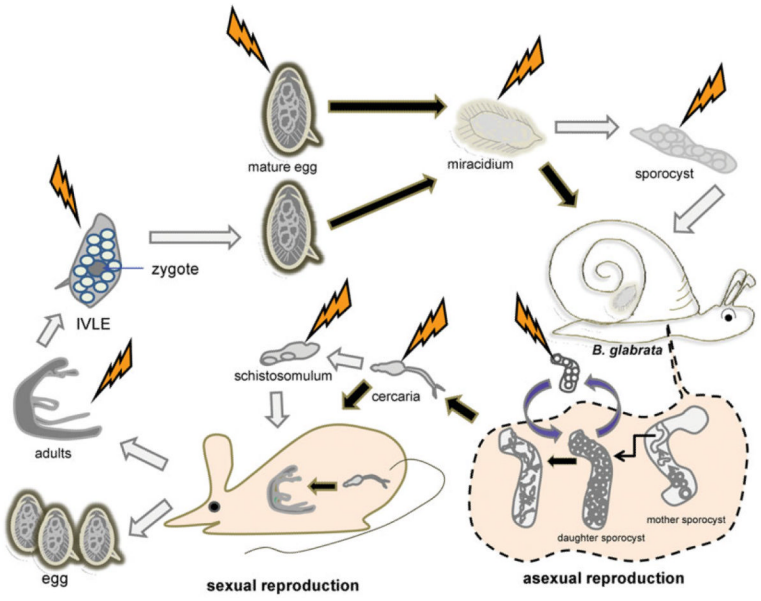

Fig. 3.

Cartoon representation of points of the developmental cycle of *Schistosoma mansoni* amenable to genetic manipulation. Both the mammalian stages involved in sexual reproduction and the snail stages with asexual reproduction are presented. Thunder bolts suggest accessible points of introduction of transgenes into the schistosome e.g. transgene delivery by electroporation, microinjection, etc. Black arrows indicate processes that occur naturally whereas white arrows represent processes that can be manipulated. Dashed line indicates events inside the snail. IVLE, *in vitro* laid eggs.