Transient Transfection of Schistosomes

Subjects: Parasitology

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The neglected tropical disease schistosomiasis is a worm infection that is caused by parasitic blood flukes. The disease is found in sub-Saharan Africa, the Middle East, Southeast Asia, and the New World. Worldwide, 240 million people are infected, and 700 million people are at risk. Schistosomiasis is a debilitating, chronic disease, and the mortality is estimated at 200,000 deaths per year. Schistosomiasis control relies on the drug praziquantel, but it does not prevent reinfection after treatment. The development of new vaccines, drugs, and diagnostic methods and the investigation of the biological basis of infectivity are, therefore, of critical importance. The development of transgenesis systems, as have been used for other pathogens, has been hampered by the complexity of the parasite and its life cycle.

schistosomiasis transfection functional genomics mobile genetic elements RNA interference RNAi genome editing CRISPR genomic safe harbour site

1. Introduction

Schistosomes belong to the phylum Platyhelminthes (flatworms) and the class Trematoda (flukes). They are digenetic blood trematodes and cause schistosomiasis in humans and other mammals by depositing eggs in the circulatory system surrounding the gut or bladder of the infected host. Five species of schistosomes have medical importance, and they can be divided into urinary schistosomes (*Schistosoma haematobium*) and intestinal schistosomes (*S. mansoni*, *S. japonicum*, *S. mekongi*, and *S. intercalatum*). This distinction is based on the pathology they cause, egg morphology, and the specific snail intermediate hosts that play an essential role in transmission.

Geographically, schistosomes are found in sub-Saharan Africa; the Middle East; Southeast Asia; and the New World in Brazil, Saint Lucia, Suriname, Venezuela, and the Caribbeans. Schistosomiasis is a neglected tropical disease with over 240 million cases worldwide, and an estimated 700 million people are at risk of infection. The disease is responsible for the loss of over 2.5 million disability-adjusted life years (DALYs), making it the third-highest global burden caused by a neglected tropical disease. The mortality of schistosomiasis is estimated at 200,000 deaths annually [1], and the morbidity (e.g., anaemia, cognitive impairment, malnutrition, and growth stunting) is in the range of 1.7–4.5 million disability-adjusted life years (DALYs) per annum [2], which is a testament to the public health significance of this disease. Schistosomiasis has been targeted by the WHO for elimination as a public health problem by 2030 [2].

The schistosome life cycle is complex and involves a sexual life-cycle phase in adult worms that reside in the definitive host and two asexual reproductive phases in the larval stages that occur in the intermediate snail host with the involvement of eggs, miracidia, mother sporocysts, daughter sporocysts, and cercariae. This asexual phase considerably increases the number of infective larvae that are released into the environment. Transmission occurs in contaminated fresh water through contact between the definitive host (e.g., humans) and the free-living larval-stage cercariae. Infected humans or bovines release eggs into the environment, which release miracidia, which later infect the snail intermediate host to complete the life cycle of the parasite. Although the adult worms reside in the circulation, the major cause of pathology is eggs of the parasite that, in the case of intestinal schistosomes, are deposited in the intestinal wall and the liver. In urinary schistosomiasis, eggs become lodged in the bladder wall, which leads to blood in the urine and inflammation.

If untreated, the disease can become chronic, with severe outcomes such as accumulation of fluid in the peritoneal cavity causing abdominal swelling; hepatosplenomegaly; portal hypertension; liver fibrosis; and, in the case of urinary schistosomiasis, secondary cancers of the bladder and death. In 5–6% of patients, the parasite may enter the central nervous system and cause further complications. The manifestations of chronic schistosomiasis are a result of a long-term battle between the host immune system and parasite evasion mechanisms. Broadly, decades of research have revealed that initial exposure triggers a Th1-biased inflammatory response, but as the parasite matures, egg secretions drive a Th2-biased, regulatory, and granulomatous response that affects liver function (see [3][4] for excellent reviews). The inflammatory milieu generated by chronic schistosomiasis also impacts the outcome of other infections present in endemic areas, and co-infected individuals have been shown to have altered antibody responses to *P. falciparum* malaria [5] and *Helicobacter pylori* [6], for example. The impact of infection extends to vaccination, and *S. mansoni*-infected individuals have reduced responses to a Hepatitis B vaccine [7]. In addition, bystander effects on non-infectious conditions such as colitis [8] and asthma [9] have also been reported in animal models.

2. Biolistics

The pioneering reports that established that transgenesis is, in principle, possible for schistosomes employed biolistic particle bombardment (gene gun) to transfer plasmids that encode reporter genes, such as jellyfish green fluorescent protein (GFP) or firefly luciferase, into schistosomes (**Figure 1**A). Davis et al. (1999) were the first to show that the biolistic transfection of adult schistosomes with a plasmid containing the luciferase gene driven by the *S. mansoni* Splice Leader RNA gene promotor or the bombardment of the parasites with a m^7G-capped, polyadenylated luciferase mRNA resulted in ~20-fold-higher expression levels in the luciferase reporter 11–40 h after transfection when compared with untreated controls [10]. Despite this success in showing that foreign genes are expressed in schistosomes, the report did not provide microscopy data to show the location of the gene expression, nor did it specify whether the worms were damaged by the procedure. After this initial report, several studies were published to address these questions by employing GFP for ease of transgene detection, extending the application of biolistic transformation to investigate schistosome protease genes [11][12][13][14][15].

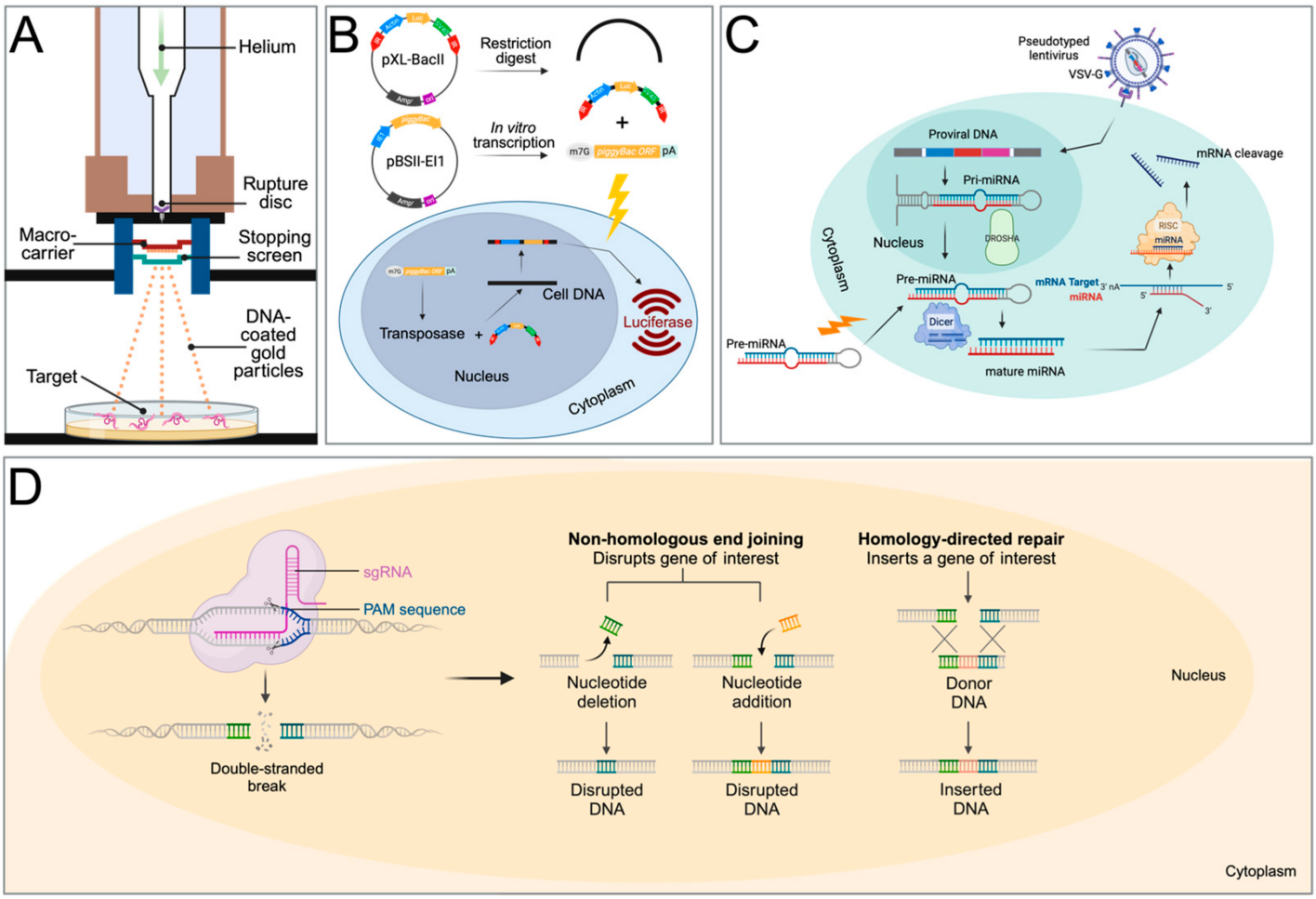

Figure 1. Transfection methods used for transgenesis in schistosomes. (**A**) Particle bombardment (gene gun) for the transfer of expression plasmid-covered gold particles. (**B**) Two-component *piggyBac* transposon system. pXL-BacII includes the *piggyBac* terminal inverted repeats (red arrows), an ampicillin resistance gene, a bacterial ColE1 replication origin, and the *S. mansoni* actin 1.1 gene promoter, driving the firefly luciferase gene. pBSII-IE1-orf is a *piggyBac* helper plasmid, providing the *piggyBac* transposase under the control of the IE1 promotor. After a restriction digest of pXL-BacII and *in vitro* transcription of the transposase from pBSII-IE1-orf, the IR-Act-Luc-IR cassette and transposase mRNA are transferred into schistosomes via electroporation, leading to luciferase expression. (**C**) Gene suppression by micro-RNA. The precursor RNA is either delivered into the cell via electroporation or with a lentiviral construct. In lentiviral delivery, DROSHA ribonuclease III cleaves primary miRNA (pri-miRNA) hairpins in the nucleus. The resulting precursor miRNA (pre-miRNA) is exported to the cytoplasm. The pre-miRNA is further processed by DICER to produce mature miRNA duplexes. RISC uses the miRNA as a template for recognising complementary mRNA. When a complementary strand is found, it activates RNase and cleaves the RNA. VSV-G: Vesicular stomatitis virus glycoprotein. (**D**) Gene editing with CRISPR. After the delivery of the Cas9 protein and sgRNA via electroporation, the target DNA is cut. Non-homologous end-joining leads to a disruption of the targeted gene (knockout), while homology-directed repair leads to gene knock-in with the help of a homology repair donor DNA. Created with BioRender.com.

First, Wippersteg et al. (2002) used a plasmid consisting of the heat shock protein 70 (*hsp70*) gene promoter and terminator sequences of *S. mansoni* in fusion with the GFP reporter gene. After the transfection of adult worms and sporocysts, GFP expression was evident on the surface of adult worms and in the internal structures of the sporocyst. By using reverse transcription PCR and Western Blot, the authors confirmed that the transgene was transcribed and translated [11].

The investigations were then extended to characterise the *S. mansoni* ER60 cysteine protease [12][13], which is associated with the excretory system (ES) of these parasites. The promoter and terminator regions of the *ER60* gene were fused with the GFP sequence to investigate the expression pattern of ER60–green fluorescent protein in sporocysts after transfection via particle bombardment. The results were consistent with the expected expression in the excretory system, with tissue-specific GFP fluorescence visible in the gland cells, protonephridia, and cytons of the larvae. This was further corroborated by co-localisation with Texas Red-BSA, which has the ability to specifically enter the excretory system of living schistosomes. The results suggested that the ER60 protease is expressed in the ES of the larvae and possibly plays a role in penetration and migration in the definitive host.

Similarly, additional schistosome proteins have been investigated using biolistic transfection [14][15][16]. Here, 5′ flanking fragments of the cathepsin L (*SmCL*), D (*SmCD*), F (*SmCF*), and B2 (*SmCB2*) peptidase genes and the calcineurin A gene (*SmCNA*) were used to construct GFP expression vectors, which were then utilised to transfect schistosome worms. The observed expression patterns of SmCL; SmCD in the gut [14]; SmCF and SmCB2 in the gut and tegument, respectively [15]; and SmCNA in the excretory system [16] was confirmed by the tissue-specific localisation of the EGFP reporter after particle bombardment.

Taken together, the studies discussed above firmly established that schistosomes are amenable to transfection and that valuable insights into the biology of this parasite could be gained using particle bombardment as a means of transfection. The main disadvantage of this method, however, is the transient nature of the transgene expression, the inability to target specific tissues of the parasite, and the difficulty in achieving germline transfection. To address this problem, the researchers' group used particle bombardment to target the germline in miracidia, the larval form of the parasite that infects the intermediate snail host and gives rise to successive generations of sporocysts and, ultimately, cercariae that infect the definitive host. The researchers were able to show that a plasmid that encodes an enhanced green fluorescent protein (EGFP) under the control of the *S. mansoni HSP70* promoter and termination elements was able to drive the expression of the reporter gene in miracidia. Transfected miracidia were able to penetrate and establish, in *Biomphalaria glabrata*, the *S. mansoni* intermediate host snail, and gold particles could be detected in the germ balls of the parasites in paraffin sections of snail tissue. In addition, the miracidia transformed into mother sporocysts inside the snails, and reporter gene activity could be determined at 10 days post-infection using RT-PCR on snail tissues [17]. This was the first research to show that it is feasible to return transgenic miracidia to the parasite’s life cycle, a crucial step in the establishment of transgenesis in schistosomes. The researchers' research was extended by Beckman et al. (2007), and in their work, after the bombardment of miracidia, the transgene was also detectable in cercariae and adults of the F0 and F1 generations [18].

3. Transposable Elements

At the same time that particle bombardment was being explored as a means of transfecting schistosomes, a different concept was gaining interest. After the release of the schistosome genome [19][20], it became apparent that these parasites have unusually large genomes. The nuclear genome comprises 363 megabases containing ~12,000 genes, which are distributed over seven pairs of autosomes and one pair of sex chromosomes, and account for about 50% of the genome [21][22]. The remaining 50% is composed of repetitive sequences, with many related to transposable elements [23].

Transposable elements (TEs), or mobile genetic elements, are DNA sequences that replicate independently in the genome. TEs consist of three major groups, with Class I representing retrotransposons, which spread through the genome via a copy/paste mechanism, creating multiple repetitive sequences throughout the genome. Class II TEs also move through the genome through a cut/paste mechanism; they are, however, not copied but rather move through the genome employing a transposase encoded by the Class II TE itself, which facilitates the cutting and pasting process. Class III TEs, also called miniature inverted-repeat transposable elements (MITEs), are relatively short elements and contain inverted repeats at each end. As most can be found in euchromatin, it has been suggested that they might be involved in regulatory functions. A comprehensive overview of eukaryotic transposable elements can be found elsewhere [24].

In schistosomes, both Class I (*Boudicca*, *Sinbad*, *Gulliver*, *Saci-1*, *Saci-2*, *Saci-3*, *Fugitive*, and *Perere*) [25][26][27] and Class II (*Merlin*, *SmTRC1*) [28][29] elements have been identified and further characterised. In bacterial artificial chromosomes prepared from *S. mansoni* genomic DNA, the researchers detected three open reading frames (ORFs) that were bounded by long terminal repeats (LTRs). The ORFs encoded sequences with homology to Group Antigen (GAG) polyproteins, reverse transcriptase, RNaseH, integrase, and a putative envelope protein, domains that are also found in retrotransposons and retroviruses [30]. The order of these domains in the genome of schistosomes was similar to that found in the gypsy/Ty3 retrotransposons, which are widely distributed among animals, plants, and fungi [31]. Analysis of the reverse transcriptase domain confirmed that the schistosome element was most closely related to *CsRn1* from the liver fluke *Clonorchis sinensis* [32] and to *kabuki* from the silk moth *Bombyx mori* [33]. The researchers named the schistosome element *Boudicca*, and copies were found throughout the genome of the parasite. In addition, mRNA transcripts of *Boudicca* were present in adult worms, sporocysts, and cercariae. This suggested that *Boudicca* is an active and transcribed element in *S. mansoni* [30].

The researchers' laboratories have also identified another new LTR retrotransposon, *Sinbad*, in schistosomes, which belongs to the *Pao/BEL*-like elements. Like *Boudicca*, the full-length *Sinbad* transposon is flanked by LTRs and contains GAG, protease, reverse transcriptase, RNAseH, and integrase motives. It is not as abundant as *Boudicca*, and analyses suggested that there are ~50 copies in the schistosome genome. Transcripts of *Sinbad* could be found in the developmental stages of schistosomes, which, again, suggested that *Sinbad* is actively transcribed. In many organisms, endogenous and exogenous TEs such as *Sleeping Beauty*, *piggyBac*, and *mariner* have been exploited to achieve transfection [34]. It was, therefore, reasonable to assume that schistosome TEs could also be used to achieve not only transient transfection as with biolistic methods but also integration into

the genome [35]. The LTRs of *Boudicca* and *Sinbad* were further studied to investigate whether they could function as promotors and, therefore, be used as vehicles for transgene introduction. The analysis revealed that the LTRs contained TATA boxes, polyadenylation signals, and direct inverted repeats. In addition, the LTRs of *Sindbad* were able to drive luciferase expression in both the forward and inverted orientations in HeLa cells [36].

Although these elements held promise for the development of a transfection system for schistosomes, their potential was not realised partly because the development of other techniques superseded the use of mobile genetic elements.

The only success using an exogenous transposable element for transfecting schistosomes was reported by Morales et al. (2007) [37]. The Class II transposon *piggyBac* from the genome of the cabbage looper moth [38] was used to deliver a *piggyBac* donor plasmid that encodes firefly luciferase under the control of schistosome gene promoters into the genome of *S. mansoni* (**Figure 1**B). Southern hybridization and retrotransposon-anchored PCR analyses showed that the *piggyBac* transposon had integrated into numerous sites within the parasite's chromosomes [37]. This was the first report to demonstrate that somatic transgenesis is possible in schistosomes.

4. RNA Interference

Sequence-specific gene silencing using double-stranded RNA (dsRNA) has been an invaluable tool for investigating gene function. Through the process of RNA interference (RNAi) or RNA silencing, dsRNA pairs with their homologous mRNA targets which prevents gene expression either via mRNA degradation or interference with protein translation (**Figure 1**C). This method has dominated functional genomics studies in schistosome research for the past 20 years.

Cathepsin B is a key cysteine protease in the digestion of haemoglobin by schistosomes and has also been used as a vaccine target. To confirm a functional role of SmCB1 in parasite survival, Skelly et al. (2003) reported the use of RNAi to inhibit *SmCB1* in adults *in vitro* and confirmed the knockdown with qPCR, immunostaining, and enzyme activity assays [39]; a later report by the same group reported greatly enhanced uptake of dsRNA after using electroporation [40]. Other groups have reported that the suppression of *SmCB1* leads to growth retardation [41] and, further, that SmCB1 knockdown could be achieved using a mouse Moloney virus vector (MMLV) [42].

Following the establishment of the RNAi technique in schistosomes, this approach has been used to investigate the function of a wide range of genes in adults, eggs, and schistosomulae. To date, the targeting of over 50 genes in *S. japonicum* and around 80 in *S. mansoni* has been reported, including a number of RNAi screen studies (discussed below). A detailed discussion of these is beyond the scope of this research. The reader is referred to reviews [43][44], and a comprehensive list of studies using RNAi may be found in the Supplementary Table S1 of [45].

RNAi In Vivo

In 2018, Li et al. reported a protocol to achieve RNAi in schistosome life-cycle stages in mammalian hosts. Initially, infected mice were treated with 10 µg of long dsRNA that targets *SjCB1* via intravenous injection. This method was found to induce a knockdown effect of 79% in male worms and 92% in female worms compared with controls, as detected using qPCR [46]. Further optimisation studies showed that the long-term suppression of gene expression could be achieved with 10 µg of dsRNA administered in six doses over 26 days. The effect of suppressing three functional genes (peroxiredoxin, Mago nashi, insulin receptor) could be confirmed with qPCR and morphological changes observed in worms isolated from treated mice. These protocols provided an important advance in facilitating the functional analysis of genes involved in worm development.

The same group went on to investigate the role of *S. japonicum* ferritin genes in maturation. Ferritin proteins store iron and might be expected to play key roles in the processing of the iron-rich blood diet of the worms. The knockdown of three ferritin genes *in vivo* revealed that *SjFer0* affected the growth and development of schistosomulae but did not affect egg production in adults. *SjFer1* and *2* did not affect growth or development [47]. More recently, investigations on the role of the signal peptidase complex (SPC), an essential part of protein translocation machinery, revealed that the *in vivo* knockdown of the *SPC25* gene led to the degeneration of reproductive organs and reduced egg production, reducing damage in the host [48].

5. Highlights of Findings from RNAi Studies

Collins et al. (2013) identified neoblast-like cells in adult schistosomes, termed proliferating somatic cells (PSCs). These cells were identified in the gut and muscle but not in reproductive organs [49]. PSCs were found to express an ortholog of fibroblast-like growth factor receptor (SmfgfrA). FGF is known in other organisms to play a role in cell proliferation, differentiation, and survival. Using labelling experiments, PSCs were shown to self-renew and differentiate. The inhibition of *SmfgfrA* using RNAi reduced the proliferation of PSCs and cell-cycle-associated gene expression. The findings of this study provide insight into how the parasites survive over a very long period in the host and how they can recover from tissue damage after sublethal doses of praziquantel, for example [50].

While most reports have focussed on a single gene or 2–3 genes in a specific pathway, wider-scale RNAi screens are required to identify potential novel drug targets. Initially, Mourão et al. (2009) performed a screen of 32 genes using synthesised dsRNA by transforming miracidia into sporocysts in the presence of dsRNA [51]. Developing larvae were monitored for phenotypic changes, including motility and altered growth, and transcript expression. Of the genes tested, 33% induced a size reduction, but only six of these genes demonstrated a significant and consistent knockdown of the targeted gene; unexpectedly, one gene was not inhibited but rather highly induced. Of the dsRNA treatments that did not result in phenotypes, only some exhibited consistently reduced transcript levels. The variable efficacy of RNAi treatment and the potential for off-target effects have demonstrated the need for optimisation and careful interpretation of data.

Stefanic et al. (2010) investigated selectivity and sensitivity using long dsRNA that targets a set of 11 genes that are known to be located in different tissues [52]. Five of the genes had previously been shown individually to be required for parasite survival. Mechanically transformed schistosomulae were incubated for up to 3 weeks. RNAi

was found to be selective, with sensitivity ranging from 40 to 75%, as determined via qPCR, but notably, no obvious phenotypes were observed.

A large-scale screen of adult *S. mansoni* targeted 2216 genes by treating adult male and female pairs with dsRNA for 30 days [53]. Substrate attachment (as a proxy of vascular attachment and normal sucker function) and the maintenance of neoblasts and germline stem cells were monitored with ethynyl deoxyuridine (EdU) labelling. The specificity of RNAi targeting was confirmed via sequencing, and the possibility of potential off-target effects was mitigated by testing additional dsRNAs. Using this method, 195 genes were identified that had attachment phenotypes, of which 121 had additional phenotypes. In addition, 66 genes resulted in stem cell maintenance defects in male testes and neoblasts. A screen of existing pharmacological agents identified compounds that could potentially target 49 of the schistosome proteins that exhibited attachment phenotypes. In vitro and *in vivo* testing led to the identification of two compounds that inhibited the proteosome (ubiquitin–proteasome (UPS) component p97), reduced ubiquitinylation *in vitro*, and prevented egg deposition *in vivo* in a mouse model. In addition, two inhibitors of protein kinases were tested and shown to cause deformation in adults and reduced survival *in vivo*. These results illustrate the utility of RNAi not only for refining drug targets but also for confirming the drug's action. Further, the schistosome UPS and tao and stk25 kinases have been shown to be druggable targets [53]. The studies using RNAi described here show substantial advances in defining new drug and vaccine targets and have permitted genome-wide screens to simultaneously identify additional targets and biochemical pathways for intervention. These studies are summarised in **Figure 2**.

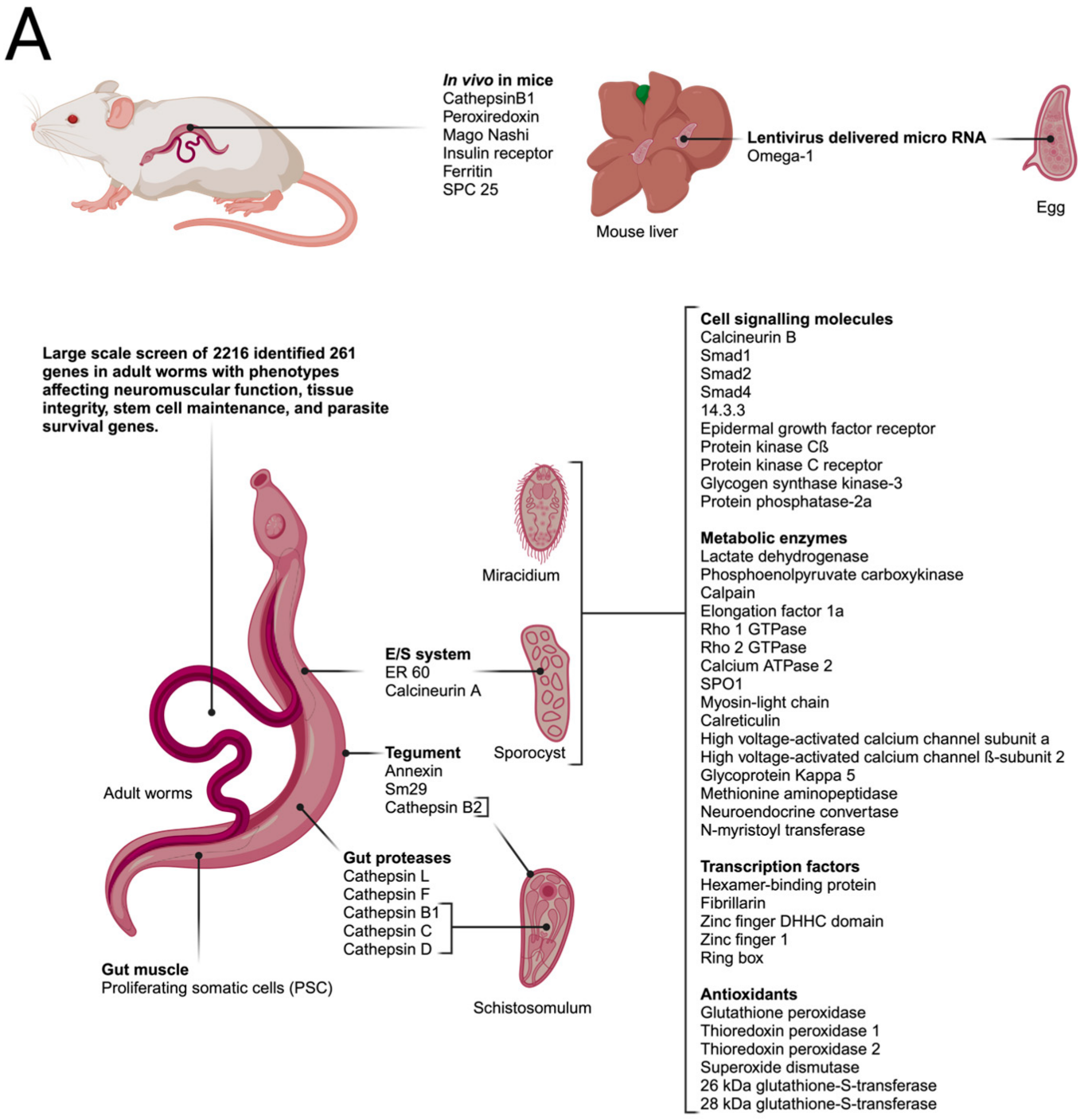
A
In vivo in mice
CathepsinB1
Peroxiredoxin
Mago Nashi
Insulin receptor
Ferritin
SPC 25
Mouse liver
Lentivirus delivered micro RNA
Omega-1
Egg
Large scale screen of 2216 identified 261 genes in adult worms with phenotypes affecting neuromuscular function, tissue integrity, stem cell maintenance, and parasite survival genes.
Miracidium
E/S system
ER 60
Calcineurin A
Sporocyst
Tegument
Annexin
Sm29
Cathepsin B2
Adult worms
Gut proteases
Cathepsin L
Cathepsin F
Cathepsin B1
Cathepsin C
Cathepsin D
Gut muscle
Proliferating somatic cells (PSC)
Schistosomulum
Cell signalling molecules
Calcineurin B
Smad1
Smad2
Smad4
14.3.3
Epidermal growth factor receptor
Protein kinase Cß
Protein kinase C receptor
Glycogen synthase kinase-3
Protein phosphatase-2a
Metabolic enzymes
Lactate dehydrogenase
Phosphoenolpyruvate carboxykinase
Calpain
Elongation factor 1a
Rho 1 GTPase
Rho 2 GTPase
Calcium ATPase 2
SPO1
Myosin-light chain
Calreticulin
High voltage-activated calcium channel subunit a
High voltage-activated calcium channel ß-subunit 2
Glycoprotein Kappa 5
Methionine aminopeptidase
Neuroendocrine convertase
N-myristoyl transferase
Transcription factors
Hexamer-binding protein
Fibrillarin
Zinc finger DHHC domain
Zinc finger 1
Ring box
Antioxidants
Glutathione peroxidase
Thioredoxin peroxidase 1
Thioredoxin peroxidase 2
Superoxide dismutase
26 kDa glutathione-S-transferase
28 kDa glutathione-S-transferase
B
Gene editing with CRISPR
Granuloma formation
Omega-1
AChe
Sj16
FgfrA
Egg
Adult worms
Drug targets and drug resistance
Smp_089290
SULT-OR

Figure 2. Transfection targets in schistosome life-cycle stages described in the text. (**A**) Genes targeted by RNA interference. (**B**) Genes targeted by gene editing using CRISPR/Cas9. Created with BioRender.com.

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