Polyethyleneimine Mediated DNA Transfection in Schistosome Parasites and Regulation of the WNT Signaling Pathway by a Dominant-Negative SmMef2

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Abstract

Schistosomiasis is a serious global problem and the second most devastating parasitic disease following malaria. Parasitic worms of the genus Schistosoma are the causative agents of schistosomiasis and infect more than 240 million people worldwide. The paucity of molecular tools to manipulate schistosome gene expression has made an understanding of genetic pathways in these parasites difficult, increasing the challenge of identifying new potential drugs for treatment. Here, we describe the use of a formulation of polyethyleneimine (PEI) as an alternative to electroporation for the efficacious transfection of genetic material into schistosome parasites. We show efficient expression of genes from a heterologous CMV promoter and from the schistosome Sm23 promoter. Using the schistosome myocyte enhancer factor 2 (SmMef2), a transcriptional activator critical for myogenesis and other developmental pathways, we describe the development of a dominant-negative form of the schistosome Mef2. Using this mutant, we provide evidence that SmMef2 may regulate genes in the WNT pathway. We also show that SmMef2 regulates its own expression levels. These data demonstrate the use of PEI to facilitate effective transfection of nucleic acids into schistosomes, aiding in the study of schistosome gene expression and regulation, and development of genetic tools for the characterization of molecular pathways in these parasites.

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Introduction

The use of transgenesis and other technological advances has had a powerful impact in the molecular characterization and functional analysis of gene function in model organisms [1,2]. However, like many parasitic worms, the natural characteristics of the schistosome (its complex life cycle involving multiple hosts, the absence of an immortalized cell line, and the inability to maintain the entire life cycle in vitro) have made in-depth genetic modifications challenging [3]. Schistosomes are the causative agents of human schistosomiasis, a parasitic disease that is endemic in 78 countries worldwide and that infects almost 240 million people [4]. In terms of morbidity and mortality, schistosomiasis is considered to be the most important helminth infection [5]. Although our knowledge of schistosome biology has increased over the last few years, the lack of simple and effective methodologies to manipulate schistosomes has slowed our understanding of schistosome molecular biology significantly behind other systems.

With the sequencing of the schistosome genome and recent updates to schistosome annotation [6,7], research has focused on the functional analysis of schistosome genes. This includes approaches to insert DNA/RNA into schistosomes and to induce gene expression. Strategies used thus far for transfection of DNA/RNA molecules include the use of particle bombardment [8–10], soaking [11–13], electroporation [14–18], chemical or lipofectamine based approaches [16,19], and viruses [20,21] (for review see [22]).

The insertion of genetic material into schistosomes by soaking in high concentrations of DNA/RNA has been successful for delivering siRNA and dsRNA [11,12,19,22,23]. This approach is straightforward; however, the transfection efficiency is highly restricted by the size of nucleic acid fragment delivered, and worm death resulting from the use of highly concentrated nucleic acids [16]. In addition, this approach is not suitable for long-term modification of the parasite genome, which requires the use of compatible sized vectors that carry information for transcription, self-amplification, and the insertion of transposable elements.

The use of biolistic particle delivery for Schistosoma mansoni (S. mansoni) transfection has been successful for several developmental stages of the parasite. These include the adult, sporocyst, and miracidia [10,24–26]. However, the square wave electroporation approach to introduce naked plasmid-based and non-plasmid-based exogenous genes into schistosomes has been more successful [8,15–17,27]. Square wave electroporation is more effective for transfection of schistosome eggs than the use of pseudotyped murine leukemia virus [21]. Consequently, electroporation has become the method of choice for schistosome transgenesis, specifically for the delivery of siRNA, dsRNA and vector based shRNA for gene silencing studies by RNAi [14–19,23,27–30]. One report, however, has suggested that the biolistic particle delivery method is more effective than electroporation for the delivery of RNA into adult worms and miracidia [8]. Nonetheless, both particle bombardment and electroporation can be damaging or even lethal to cells and...
Author Summary

Schistosomiasis is a global disease infecting more than 240 million people worldwide and is ranked second only to malaria in global health importance. The causative agents of human schistosomiasis are parasitic worms that ingest red blood cells and can live for decades producing hundreds of eggs daily. There is one primary drug for treatment of schistosomiasis, but its use for over 30 years has raised concern over the development of drug resistance and thus created a need for new drugs. A challenge to the rational development of effective anti-schistosomal therapies has been the difficulty in manipulating schistosome gene expression, and thus a limitation in our understanding of schistosome gene function. Here, we present a new and straightforward method for inserting genes into schistosomes and expressing them in addition, to our knowledge we provide the first example of dominant negative gene expression to modify transcriptional regulation using a molecular genetics approach to study this globally important parasite.

Materials and Methods

Animal preparation

Cercariae of *S. mansoni* NMRI strain (NR-21962) or strain PR-1 (NR-21961) were shed from the infected *B. glabrata* snails obtained from the Biomedi Research Institute (Rockville, MD) and transformed into schistosomula as previously described [48,49]. Seven to ten thousand schistosomula were cultured in complete RPMI medium (RPMI, 5% Fetal Bovine Serum, 1× Pen/Strep) per well in 12-well cell culture plates (Greiner Bio-One, Orlando, FL) at 37°C and 5% CO₂ for 4 hours before being utilized for transfection. For the longevity experiment, modified Basch Medium 169 (Basch Medium 1695, Fetal Bovine Serum, 1× Pen/Strep) was used for the first three days of culture. After three days, the media was changed and replaced with complete Basch Medium [48].

Construction of vectors for transfection

DNA primers were designed and ordered from Integrated DNA Technologies (IDT, Coralville, IA). Subcloning was performed using the In-Fusion HD Cloning kit (Clontech, Mountainview, CA). The full transcript of the mCherry gene from the transposon vector pKm225 (GenBank: HQ396839.1), the first 399 bp (133 amino acids) of *SmMe2*, and the wild-type *SmMe2* [40] (NCBI accession number: JN900476) were amplified by PCR using Phusion High-Fidelity DNA Polymerase (NEB, Ipswich, MA) with three sets of primers: oEJ1020 forward (5’-TCA GTC GTG TTA CCT CTA GAA TGG TGA AGG GCC GCG AGG AG) and oEJ1021 reverse (5’-GCC CGG GTC GTG TTA CCT CTA GAA TGG TGA AGG GCC GCG AGG AG) and oEJ1026 forward (5’-CAG AGT GTA CCT CTA GAC TGG ACA GGG CAC TTA ACT CAT CA-3’) and oEJ1027 reverse (5’-GCG TTA CAC GTC GTA CAG CAT CAT-3’). The mCherry amplicon was subcloned into the pCI-neo plasmid (Promega, Madison, WI) at the XhoI and NotI sites. The wild-type *SmMe2* (*SmMe2*) amplicon was subcloned into the pCI-neo plasmid at the XhoI and NotI sites. Constructs were transformed into chemically competent One Shot TOP10 cells (Invitrogen, Carlsbad, CA). The mCherry reporter plasmid (pEJ1175, *SmMe2*, 133 expression plasmid [pEJ1181]) and *SmMe2* expression plasmid (pLS068) (Figure 1) were purified using the Nucleospin Plasmid miniprep kit (Clontech, Mountainview, CA) and verified by restriction digestion analysis.

Plasmid pEJ1116 contains 2000 base pairs of the *Sm23* upstream activation sequence (UAS) regulating the expression of mCherry. To make this construct, the mCherry transcript was amplified from plasmid pEJ604 using primers oJM16 forward (5’-CGT TTG AAA GTA TGG GAT CCA TGG TGA AGG GCC GCG AGG AG) and oJM17 reverse (5’-CTG TTT TCT TGT CAG TGT CCT GAG TTA CTT GGT GAT CTC CAT GCC), then subcloned between the BamHI and PsI sites in the pGBK17 vector (Clontech, Mountainview, CA). The 200 base pair region containing the upstream activation sequence of *Sm23* was amplified from schistosomula genomic DNA using oligos oJM12 forward (5’-ATG GAG GAC GCC GAA TTC CCG GGA CCC GAA CAC TAT AGT GTG ATG CAG) and oJM13 reverse (5’-CCG CTG CAG GTC GAG GAT CAC ACA TCT TGA AAC GGG ACA CAA TGC), then subcloned into the XmaI and BamHI sites of the same vector to make plasmid pEJ1116. To review, plasmid pEJ1116 contains the 2000 base pair UAS of the *Sm23* promoter, followed by the mCherry reporter gene (Figure 1B).
Transfection and gene transcript analysis

The transfection of vector pEJ1175 into parasites was tested by standard PCR using Taq DNA Polymerase (NEB, Ipswich, MA). DNA oligos oEJ1022 (5'-TAA CAT GGC CAT CAT GAA GGA GGT TCC-3') and reverse oligo oEJ1019 (5'-ATA CTT TCT CCG GAG GAC CA-3') were added to amplify a 237 bp base pair DNA fragment including a partial mCherry and a partial neomycin sequence within the plasmid (Figure 1).

Total RNA from each DNase treated sample was used to make cDNA by RNA reverse transcription reaction using SuperScript III Reverse Transcriptase, RNase OUT and oligo (dT)12–18 (Invitrogen, Carlsbad, CA) at 37°C for another 20 min to digest mRNA thoroughly. Both reverse transcriptase and RNase H were inactivated by incubation at 70°C for 15 min. The quality of cDNA was tested by PCR amplification of a 374 bp Sm23 gene fragment using primers oJM18 forward (5'-CGT TTG AAA GTA TGG GAT CCA TGG CAA CGT TGG GTA CTG GTA TGC-3') and oJM20 reverse (5'-GTA CTT GCT GCC CAA GGA-3') and DNA plasmid (4.8 μg) and DNA plasmid (4.8 μg) as template in a 60 μL qRT-PCR reaction volume. A no reverse transcriptase control was used in all experiments. The reaction was performed at 50°C for 40 min and then treated with 10 U RNase H (New England Biolabs, Ipswich, MA) at 37°C for another 20 min to digest mRNA thoroughly. Both reverse transcriptase and RNase H were inactivated by incubation at 70°C for 15 min.

To analyze the expression of the mCherry gene under control of a human cytomegalovirus (CMV) promoter (pEJ1175, Figure 1A) and Sm23 promoter (pEJ1116, Figure 1B), qRT-PCR was carried out using primers oEJ1022 forward and oEJ1023 reverse (5'-TAA CAT GGC CAT CAT GAA GGA GGT TCC-3' and reverse oligo oEJ1019 (5'-ATA CTT TCT CCG GAG GAC CA-3') to clone a 192 bp mCherry gene fragment from 60 ng cDNA template.

Two sets of primers were designed for detection of SmMef2 transcripts by qRT-PCR. The first set of primers measure SmMef2 transcripts. The other primers sets were designed for detection of four SmMef2 transcripts. All primers were verified by Primer3 online software (http://frodo.wi.mit.edu/, Supplementary Table S1).

Sixty nanograms of cDNA from parasites treated with both PEI and pEJ1181 was used as template in a 60 μL qRT-PCR reaction with Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and used for the transfection of schistosomes. PEI and DNA plasmid (4.8 μg) was received as a gift from Dr. Puthupparampil Scaria (AparnaBio, Rockville, MD), and used for the transfection of schistosomes. PEI (7.2 μg) and DNA plasmid (4.8 μg), either pEJ1175, pEJ1181, or pEJ1116, were diluted in 1 mL of complete RPMI [49], separately. Then, the 1 mL PEI solution was added to the 1 mL DNA solution drop by drop to make a 2 mL PEI/DNA mixture with a PEI and DNA phosphate (N/P) ratio of either 6:1 or 11:1, followed by 10–15 sec vigorous vortexing. The PEI/DNA/RPMI solution was incubated at 37°C for 30 min to allow the PEI and DNA to form a nanoparticle complex. The complete RPMI from the 4 h schistosomula culture was carefully removed, leaving the schistosomula at the bottom of the culture well. Two mL of pre-warmed PEI/DNA solution was then added to the plate well and schistosomula were grown in the transfection mixture for another 40 h at 37°C and in 5% CO2. All above procedures were performed under sterile conditions. For each DNA transfection experiment, schistosomula were cultured in complete RPMI medium lacking PEI or DNA, or without both as negative controls.

Total DNA/RNA extraction from schistosomula

At 40 hours post-transfection, the supernatant was removed from 4 h schistosomula by centrifuging the parasites at 1,500 × g for 2 min. Recovered parasites were washed with 1.5 mL of 1× phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4 and 1.47 mM KH2PO4 at a final pH of 7.4) twice to remove potentially contaminating residual DNA and PEI remaining in the tube, incubated for 15 minutes in 50 units of DNase I to remove any remaining external DNA, and resuspended in 1× phosphate buffered saline.

Total DNA was purified using phenol-chloroform (Thermo Scientific, Waltham, MA). Five mg/mL glycogen (Invitrogen, Carlsbad, CA) and 3 M sodium acetate were added during the purification to increase the yields of DNA.

Total RNA was isolated following the standard manufacturer’s protocol for the PureLink RNA Mini Kit using TriZol reagent (Invitrogen, Carlsbad, CA). DNase I digestion was performed to eliminate DNA contamination.

RNA and DNA were quantified on a Nanodrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA) and the quality was verified by visualization on agarose gels.

PEI-mediated plasmid transfection

In VitroPlex-Express-Parasite (Cat # IVTP-ExPA-002), a formulation of PEI optimized for nucleic acid delivery into parasites, was received as a gift from Dr. Puthupparampil Scaria (AparnaBio, Rockville, MD), and used for the transfection of schistosomes. PEI (7.2 μg) and DNA plasmid (4.8 μg), either pEJ1175, pEJ1181, or pEJ1116, were diluted in 1 mL of complete RPMI [49], separately. Then, the 1 mL PEI solution was added to the 1 mL DNA solution drop by drop to make a 2 mL PEI/DNA mixture with a PEI and DNA phosphate (N/P) ratio of either 6:1 or 11:1, followed by 10–15 sec vigorous vortexing. The PEI/DNA/RPMI solution was incubated at 37°C for 30 min to allow the PEI and DNA to form a nanoparticle complex. The complete RPMI from the 4 h schistosomula culture was carefully removed, leaving the schistosomula at the bottom of the culture well. Two mL of pre-warmed PEI/DNA solution was then added to the plate well and schistosomula were grown in the transfection mixture for another 40 h at 37°C and in 5% CO2. All above procedures were performed under sterile conditions. For each DNA transfection experiment, schistosomula were cultured in complete RPMI medium lacking PEI or DNA, or without both as negative controls.

**Figure 1. Expression constructs used for schistosome transfection.** (A) The mCherry reporter gene was cloned into the BamHI site of the pCI-neo vector (Promega) to make plasmid pEJ1175. DNA oligos used for amplification by PCR or RT-PCR are shown as a forward arrow (a) or reverse arrows (b and c) representing forward oligo oEJ1022 (a), and reverse oligos oEJ1023 (b) and oEJ1019 (c). (B) 2000 base pairs of the Sm23 UAS was used to control expression of the mCherry and the Sm23 genes. These genes were cloned into the 7.4 kb pGBKTK7 vector to make plasmid pEJ1116. (C) The N-terminal 133 amino acids of SmMef2 are regulated by the CMV promoter and were cloned to make plasmid pEJ1181. The N-terminus of SmMef2 contains the DNA binding domain, but not its C-terminal transactivation domain. (D) The wild-type SmMef2, regulated by the CMV promoter, was cloned to make plasmid (pLS068). DNA oligos (d) and (e) are used for detection of SmMef2 transcript by qRT-PCR, while oligos (f) and (g) are used for specifically measuring wt SmMef2 transcript in qRT-PCR reactions.

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**PEI Mediated DNA Transfection in Schistosomes**

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curve analysis of each pair of primers showed that only one specific
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qRT-PCR, to differentiate between the non-specific gene ampli-
stosomes transfected with pEJ1175 and pLS068 were quantified by
controls (No RT and DNA only treatment) were run in parallel.
result of overexpression SmMef2,133, respectively. Negative
cells were then added with 5
DNA was extracted from each group and
promoter (Figure 1A). As negative controls for transfection, equal
DNA contained the mCherry gene regulated by a strong CMV
promoter (Figure 1A). Seven to ten thousand schistosomula transfected with one of the
two c-Myc tagged plasmids and were harvested 44 h after cercarial
transformation. Samples transfected with pEJ1175 were used as a
control. Schistosomula were washed with 1 PBS twice and resuspended in the lysis buffer (20 mM Tris-HCl, 200 mM
NaCl, 1% PMSF and 1% Halt Protease Inhibitor Cocktail; Thermo
Scientific, Waltham, MA), followed by 6 x sonication of 15 s pulses,
30% amplitude with 1 min interval between each pulse. Cell lysate
was then added with 5 SDS loading buffer and boiled at 100°C
for 10 min and incubation for 5 min on ice. Fifty microliters of the supernatant from each cell lysate was resolved on NuPAGE 4–12%
Bio-Tris ready-made gels (Invitrogen, CA). The protein was trans-
ferred to a nitrocellulose membrane (Thermo Scientific, MA) and
blocked in 5% milk. The specific expression of c-Myc tag protein was detected by the mouse monoclonal IgG, c-Myc (Myc:A7) primary antibody and a goat anti-mouse IgG-HRP secondary antibody (Santa Cruz Biotec-
ology, CA). Similarly, mCherry protein expression was detected by the mouse monoclonal IgG2a primary antibody (Novus Biologicals, Littleton, CO) and the goat
anti-mouse IgG-HRP secondary antibody (Santa Cruz Biotechnology, CA), and assayed by western blot analysis.

Protein expression and Western blot analysis
Both SmMef2,133 and the wild type SmMef2 gene with a c-Myc
tag at the 5-prime end were amplified from pEJ1114 [49] and
subcloned into pCI-neo vector using the methods described above.
Seven to ten thousand schistosomula transfected with one of the
two c-Myc tagged plasmids and were harvested 44 h after cercarial transformation. Samples transfected with pEJ1175 were used as a
control. Schistosomula were washed with 1 PBS twice and resuspended in the lysis buffer (20 mM Tris-HCl, 200 mM
NaCl, 1% PMSF and 1% Halt Protease Inhibitor Cocktail; Thermo
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anti-mouse IgG-HRP secondary antibody (Santa Cruz Biotechnology, CA), and assayed by western blot analysis.

Results
PEI facilitates the transfection of DNA in schistosomes
PEI has been used successfully for gene delivery in mammalian cells in vitro and in vitro [51–53], and recently in the snail, Biomphalaria glabrata [38]. The success of gene delivery using PEI in snails inspired us to ask whether PEI could be used as an alternative to electroporation to transport genetic material into schisto-
somes. To test this possibility, we incubated 4-hour schistosomula for 40 hours in a PEI/plasmid DNA mix in complete RPMI
(Figure 2, Lanes 3 and 6, see Material and Methods). The plasmid DNA contained the mCherry gene regulated by a strong CMV promoter (Figure 1A). As negative controls for transfection, equal numbers of schistosomula were cultured in RPMI medium containing (1) only PEI, (2) only DNA plasmid, or (3) only schisto-
somes, no PEI and no DNA (Figure 2, Lanes 2–4). We examined the efficacy of the use of PEI for the introduction of plasmid DNA into schistosomula by using different N/P ratios, 6:1 (Lane 5) and
11:1 (Lane 6). Forty hours after transfection, we treated all schi-
ostomula with DNase to remove any contaminating external DNA. Total schistosome DNA was extracted from each group and
used for standard PCR analysis to test for the presence of the 2,377 base pair fragment stretching from the mCherry gene to the neomycin gene of plasmid pEJ1175 (Figure 1A). We found that the expected 2,377 base pair fragment was amplified only from samples containing both PEI and plasmid DNA (Figure 2, Lanes 5
and 6), whereas all negative control samples (Lanes 2–4) had no product. This result is consistent and reproducible (n=5), and demonstrates that PEI can be used to introduce plasmid DNA into schistosomes.

After treatment with PEI, we observed the parasites by light microscopy. Under some conditions, PEI can have potential toxic effects to human cells [54]. To test for potential lethality to schisto-
somes due to PEI exposure, we incubated approximately 8,000 schistosomula in the 2 mL PEI/DNA mix in complete RPMI media for two days. We found no significant differences in viability between schistosomula incubated with PEI (with plasmid DNA) and schistosomula grown without PEI in the medium (Supple-
mentary Table S2). To assay viability, we pipeted the schistosom-
ula in media and removed 5–10% of the parasite culture after 1 hour, 1 day, and 2 days. The schistosomula were allowed to settle briefly and were counted. We counted the schistosomula that settled on the culture dish and that were motile as alive, but schistosomula that did not settle on the plate, were not observed to be motile, or that appeared to lyse, were counted as dead. We rationalized that if PEI is deleterious, then under stressful condi-
tions where the schistosomula were crowded due to large numbers, toxicity might be exacerbated. Our data indicate that lethality due to exposure to PEI is not a major concern for culturing schisto-
sona at the concentrations utilized in these experiments.

The nitrogen/phosphate (N/P) molar ratio of PEI/DNA com-
xplexes is an important factor for effective transfection of DNA into mammalian cells [55,56]. An N/P ratio of 6:1 is optimal for the transfection of most mammalian cells [57]. Using PCR to amplify a 2,377 base pair sequence from plasmid pEJ1175 (Figure 1), we
assessed whether a change in the ratio of PEI to DNA would affect the efficacy of the transfection of the plasmid DNA in schistosomes. We assayed two N/P ratios, 6:1 PEI/DNA, and 11:1 PEI/DNA, and both ratios were found to be effective for transfection of DNA into schistosomes (Figure 2, Lanes 5 and 6, respectively).

CMV and Sm23 promoters can induce gene expression from a plasmid in PEI transfected schistosomula
Since PEI can be used to insert DNA into schistosomes, we
assayed whether the mCherry reporter gene, under control of a CMV promoter, could be expressed from a plasmid in transfected schistosomes. Previously, schistosomes transfected using either particle bombardment or electroporation showed that the CMV promoter is capable of inducing heterologous gene expression in these parasites [17,58]. Thus, the use of CMV as a testable promoter was considered valid. To test for expression from the CMV promoter after PEI mediated transfection, DNase treated total RNA was extracted from schistosomula after treatment with or without PEI, and subsequently followed by two-step reverse transcription PCR (RT-PCR) to amplify a 192 base pair fragment of the mCherry RNA transcript (Figure 1A). Since mCherry is not endogenous in schistosomes, only parasites that have been suc-
cessfully transfected with the plasmid will be capable of expressing mCherry. Our RT-PCR analysis confirms that the CMV promoter is sufficient to induce transcription of the mCherry reporter gene in schistosomula (Figure 3A, Lane 2), but not in the
negative control sample (Figure 3A, Lane 4). No product was observed in a control sample tested without reverse transcriptase (data not shown).

We next evaluated whether a larger DNA plasmid could be transfected into schistosomes, and assayed the expression of a reporter gene on the plasmid directed by the schistosome Sm23 promoter. Sm23 is an integral membrane protein in schistosomes [59,60]. We cloned 2000 base pairs of the Sm23 upstream activation sequence containing the Sm23 promoter into the vector pGBKKT7 (Clontech). Directly under control of the Sm23 promoter, we subcloned the mCherry gene (Figure 1B) to produce the 10.4 kb plasmid, pEJ1116 (Figure 1B). We showed that PEI could be used for transfection of the smaller 6.2 kb mCherry vector pEJ1175 (Figure 1A). Here, we evaluated PEI for the transfection of a larger 10.4 kb DNA plasmid. We transfected the 10.4 kb plasmid pEJ1116 into 4-hour schistosomula. After transfection of the 10.4 kb plasmid pEJ1116 into 4-hr schistosomula, we assayed for the amplification of a 192 base pair mCherry product to test for the expression of the mCherry transcript, as described above. Expression of the mCherry transcript can only occur in schistosomula that are successfully transfected and then, only if the plasmid based promoter, Sm23, is functional. After RT-PCR analysis, we found that mCherry is expressed from the Sm23 promoter on the 10.4 kb plasmid, demonstrating that PEI is sufficient to aid in the transfection of large plasmids into schistosomes, and that the Sm23 UAS is sufficient for gene expression from a plasmid (Figure 3A, Lane 3).

We investigated whether schistosomes transfected with a plasmid transcribing mCherry, under control of the CMV promoter, were able to express the mCherry protein using Western blot analysis (Figure 3B). Using an antibody against mCherry, we observed a 28 kD mCherry protein in schistosomes expressing mCherry from the CMV promoter (Figure 3B, Lane 1); but this was not observed in untransformed schistosomes (Figure 3B, Lane 2).

Schistosome Mef2 is transcriptionally autoregulated and regulates WNT gene transcript levels

We previously identified and characterized Mef2 in schistosomes (SmMef2) [40], a conserved transcriptional activator that is essential for myogenesis in Drosophila [41]. Mef2 also has diverse functions regulating cellular differentiation, morphogenesis and proliferation [61,62]. Recent studies in mice provide evidence that Mef2 proteins can modulate signaling of the WNT pathway during skeletal muscle regeneration [63]. We previously reported that there were potential Mef2 DNA binding sites within 500 bp of the translation start sites of two schistosome genes encoding WNT homologs: Smp_152900, encoding for Wnt1, and Smp_167140, which we assert, based on conserved sequence analysis, encodes Wnt2 [64].

The ability to easily transfect and induce gene expression in schistosomes with low lethality using PEI, and the developmental question of whether Mef2 plays a role in regulating genes in the WNT pathway, provided an opportunity to test whether schistosome transfection with PEI could be used as a genetic tool to dissect basic gene functions in schistosomes. To address this, we propounded the idea that expression of a SmMef2 mutant that can (1) bind DNA, but (2) be unable to efficiently induce Mef2 transcriptional target genes, could potentially interfere with normal SmMef2 activator function in vivo by acting as a competitive inhibitor and act as a potential genetic dominant negative in schistosomes. SmMef2 has a N-terminal DNA binding and a C-terminal transactivation domain. We removed the C-terminal transactivation domain of SmMef2, producing a truncation mutant comprising the first 133 amino acids containing the MADS box and Mef2 DNA binding domains to make SmMef2,133. We cloned the truncated schistosome SmMef2,133 gene so that its expression was controlled by the strong CMV promoter (Figure 1C) and transfected schistosom-
ula with this construct as before. After 40 hours, we extracted RNA and used qRT-PCR to compare SmMef2,133 transcript levels to an untransfected control. The control was incubated with the Mef2,133 plasmid without PEI. We found that SmMef2 levels were increased twenty-fold higher than SmMef2 levels in the untransfected control (Figure 4 A), demonstrating significant upregulation of the SmMef2 transcript.

Since the promoters of SmWnt1 and SmWnt2 genes have Mef2 binding sequences, we tested whether SmMef2,133 overexpression has an effect on the transcript levels of SmWnt1 and SmWnt2 by qRT-PCR. When SmMef2,133 is overexpressed, we found that Wnt1 transcript levels are downregulated some 2 fold (Figure 5A), and Wnt2 transcript levels were downregulated more than 5 fold compared to the untransfected control (Figure 5B). As a negative control, when mCherry was overexpressed we observed no significant changes in Wnt1 or Wnt2 transcript levels (Figure 5 A,B). When we tested a muscle LIM gene (Smp_143130) and a TGF beta family gene (Smp_063190) that have a potential Mef2 binding site, we found no significant difference in transcript levels (data not shown, Supplementary Table 1).

SmMef2 levels are highest in 4-hour schistosomula relative sporocysts, cercariae, and adult worms [40]. We cloned the
SmMef2 gene under control of the CMV promoter, as was previously described for the truncation mutant, SmMef2,133, and overexpressed SmMef2. To distinguish between expression of SmMef2 and the mutant SmMef2,133, we designed DNA oligonucleotides that recognize SmMef2 (Figure 1D, oligonucleotides f and g) but that do not recognize SmMef2,133 transcript. DNA oligonucleotides that recognize sequences in SmMef2,133 (Figure 1D, oligonucleotides d and e) also recognize SmMef2 sequences. We found that expression of SmMef2 was elevated 30-fold relative to the control (Figure 4B), whereas overexpression of the mCherry negative control had no effect on SmMef2 transcript levels. A second pair of oligonucleotides (Figure 1D, oligonucleotides d and e) that recognize sequences in SmMef2,133, and SmMef2, showed a 25 fold increase in Mef2 transcript levels (Figure 4D), indicating the confidence level of the qRT-PCR data.

We then assayed if overexpression of SmMef2 could have a positive effect on WNT levels. We found a very slight increase in Wnt1 transcript, but no significant change in Wnt2 transcript levels. The Mef2 protein is reported to positively regulate its own transcription [65]. We investigated whether SmMef2 was capable of regulating its transcription levels in schistosomes. To address this genetically, we overexpressed the truncated mutant, SmMef2,133, and measured SmMef2 levels by qRT-PCR using oligonucleotide pairs that distinguish SmMef2 transcript from SmMef2,133 transcript as described (Figure 1D). We found that overexpression of SmMef2,133 resulted in a 3-fold decrease of SmMef2 transcript (Figure 4C), strongly suggesting a role for Mef2 positively regulating its expression.

We assayed for distinct changes in viability between schistosomula expressing the dominant negative mutant, SmMef2. One thousand schistosomula were transfected with plasmid expressing SmMef2,133, SmMef2 or a nonspecific control, mCherry. These were grown for 7 days in Basch medium (see Material and Methods). After 7 days, all worms were quantified. We found 640, 550, and 620 schistosomula transformed with SmMef2,133, SmMef2, and the mCherry control respectively remained alive. Thus, we observed no significant differences in survival rate.

SmMef2 protein is expressed in schistosomes

SmMef2 and SmMef2,133 transcript levels are upregulated when expressed from the CMV promoter. We addressed whether this expression led to production of protein. To assay protein expression from the reporter construct in schistosomes, we added a c-Myc tag sequence to the 5-prime ends of the truncated mutant SmMef2,133 and the wt SmMef2 genes. We then extracted protein from schistosomula transfected with the plasmids expressing myc tagged SmMef2 and myc-tagged SmMef2,133 at 40 h post-transfection, and we assayed protein expression by Western blot analysis. Both c-Myc tagged SmMef2,133 (18.3 kDa) and c-Myc tagged SmMef2 (77.1 kDa) were detected by Western analysis and visualized using a gel documentation system (Figure 6). These data confirm that exogenous gene transcripts are translated into protein.

![Figure 4. SmMef2 can autoregulate its transcript levels.](image-url)
Discussion

Here, we have shown 1) that PEI facilitates the transfection of nucleic acids into schistosomes, and 2) that it facilitates the molecular genetic analysis of signaling and transcriptional pathways in schistosomes, addressed here by assessing SmMef2 function on SmWnt1 and SmWnt2 genes as proof of principle. 3) We provide an example of dominant-negative gene expression in schistosomes, and 4) provide evidence that SmMef2 is autoregulatory, and show data supporting its role in the regulation of the WNT pathway.

The idea to examine PEI for the transfection of DNA into schistosomes was inspired by the report that showed it was a useful agent for the successful transfection of *B. glabrata* snails [38]. PEI is an established transfection agent for individual mammalian cells [33], for tissue culture [66], and for tumor therapy [67]. The transfection of live snails led us to test whether it could also be efficacious as a transfection agent in schistosomes. We found that PEI (Aparna Biosciences, Rockville, MD) is extremely effective for the transfer of nucleic acids into schistosomula. DNA plasmids up to 10.4 kb in size were successfully introduced into schistosomula and were functional for transcription of a heterologous reporter. We recommend the use of PEI as an alternative to the aforementioned transfection approaches previously used in schistosomula. Although electroporation has been the most widely used method for transfection in schistosomes, electroporation can lead to significant mortality after passage of electrical amperage into worms. It also requires the purchase of an electroporator and cuvettes. The schistosomes must be transferred into cuvettes with nucleic acids in minimal salt solution to avoid arcing, prior to *in vitro* or *in vivo* culturing, increasing the possibility of contamination. Our data suggests that PEI at the levels used and described here does not increase lethality to transfected parasites. In addition, the use of PEI as a transfection agent is straightforward, requiring the addition of PEI and less than 10 μg of DNA in our studies. Mechanistically, DNA and PEI are incubated in the same culture medium containing the parasites, making the technique simple.

We tested two different promoters for expression in schistosomes after transfection using PEI- the CMV promoter, and the schistosome Sm23 promoter. Both promoters were capable of inducing gene expression from plasmids when tested 2 days after transfection was initiated. Initially, we used mCherry as a reporter gene for expression under the premise that we could visually screen for transfected schistosomula under a microscope and that
we could be able to determine the exact efficiency of transfection by quantifying the percentage of fluorescent schistosomes. We found that the background autofluorescence of schistosomes masked consistent discrimination between transfected and untransfected schistosomes. Since the PEI does not specifically localize DNA during transfection to a discrete locations in the parasite (i.e. the gut, the nerves, schistosome surface) and there is as yet no organelle specific reporter described in schistosomes, it is possible that diffuse fluorescence of the mCherry reporter cannot be observed visually using our methods. Thus, we assayed transfection and reporter activity by directly quantifying schistosome RNA levels in the transfected parasites.

The ease of this approach to transfect schistosomes in combination with our interest in transcriptional regulation and our previous work on SmMef2 in schistosomes, stimulated us to inquire if we could develop a genetic model to investigate basic biological questions on SmMef2 gene expression in schistosomes. We predicted that expression of a truncated SmMef2 protein, that contains the DNA binding domain but no transactivation domain (SmMef2,133), could antagonize or compete with wild-type SmMef2 for binding to SmMef2 transcriptional targets, and potentially interfere with expression of SmMef2 target genes. We identified potential Mef2 binding elements in several schistosome promoters, including SmWnt1 and SmWnt2 [40] When we overexpressed the truncated SmMef2,133, we found that both SmWnt1 and SmWnt2 transcript levels were reduced by two-fold and five-fold, respectively. We similarly overexpressed a control mCherry gene to test whether overexpression of any gene could lead to general down regulation of schistosome gene expression, but observed no change in Wnt1 or Wnt2 transcript levels. This indicates that Wnt1 and Wnt2 transcription is regulated by SmMef2, either directly or indirectly. Although, the presence of Mef2 binding sites in the promoters of SmWnt1 and SmWnt2 might suggest that this interaction is direct.

We overexpressed SmMef2 to assay whether elevated levels of SmMef2 levels could lead to an increase in Wnt1 or Wnt2 transcript levels. We found an indication of change in Wnt1 transcript levels. Mef2 transcript levels are highest in schistosomula compared to sporocysts, cercariae, or adults. It could be that the normal high expression level of SmMef2 at this stage saturates Mef2 targets and increasing Mef2 levels higher has little effect. This rationale corresponds to work done on myoblast cells where a dominant negative version of Mef2 reduces MyoD induced myogenic colony formation, but overexpression of Mef2 had no effect on myogenic

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**Figure 7. A simplified expanded model Mef2 gene regulation.** Regulation of Mef2 is complex. Mef2 expression is induced by myogenin, and Mef2 protein induces myogenin and its own expression. Mef2 also regulates genes necessary for muscle differentiation and the microRNA miR-1. miR-1, in turn, inhibits the histone deacetylase HDAC4 from repressing Mef2, allowing increased Mef2 expression. Mef2 also induces HDAC9 and miR-92b, which work to negatively repress Mef2 expression and Mef2 activator function, respectively. The upper panel in blue describes known Mef2 interactions in other systems. The lower panel in pink highlights a role for SmMef2 in the regulation of SmWnt1 and SmWnt2, and in SmMef2 autoregulation in schistosomes as described in this report.

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conversion [68]. Alternatively, it could simply be that Mef2 requires an interacting factor for the expression of some Mef2 targets or post-translational modification of SmMef2, which has been established for several Mef2 target genes [69–73].

We also found that SmMef2 was capable of regulating its own transcript levels in whole schistosomes, which has been reported previously in mammalian cell culture [65]. When the dominant negative SmMef2,133 was overexpressed, SmMef2 transcript levels were reduced three fold, showing that SmMef2 regulates its own transcription. In Drosophila, Mef2 interacts with microRNAs (miRNA), specifically miR-1, that targets and reduces the mRNA stability and translation of class II histone deacetylases (HDACs), specifically HDAC4. HDAC4 is a transcriptional repressor of muscle specific genes [74]. In this model, downregulation of Mef2 would reduce expression of miR-1. With reduced miR-1, HDAC levels are not suppressed and in turn can repress of SmMef2 (Figure 7). Thus, overexpression of Mef2 could presumably lead to an exponential increase in its expression. On the contrary, Mef2 also induces targets that negatively regulate its levels. Mef2 activates the miR-92b, a recently identified microRNA that represses Mef2 transcript [75]. Similarly, Mef2 can activate HDAC9. HDAC9 in turn interacts with Mef2 proteins to repress Mef2 transcriptional ability [76]. Thus, Mef2 forms a feedback loop that maintains an equilibrium in Mef2 expression and Mef2 target induction. This may also explain why overexpression of SmMef2 did not produce a significant increase in Wnt1 and Wnt 2 expression levels.

Since Mef2 is important for myogenesis and neuron survival in other organisms, we predicted that overexpression of SmMef2,133 would produce a phenotypically distinct mutant due to lack of muscle development or a reduction in neuronal survival. However, after microscopic analysis, we were unable to identify a visual physical difference in either motility or in shape between schistosomes overexpressing SmMef2,133, or SmMef2, or a mCherry control schistosomula, even after 7 days. Nor did we find a quantitative change in a predicted muscle Lim gene (Smp_143130) or a TGF beta family gene (Smp_152900) at two days, which have a potential SmMef2 binding site (data not shown). One reason for this could be that factors other than SmMef2 can participate in muscle or neuromuscular development [41,77]. Alternatively, the transcription activation function of SmMef2 protein function may be inhibited by a HDAC9-like protein in schistosome preventing induction, or simply that the schistosomula should be cultured for a longer periods to observe any gross phenotypic changes contributed to overexpression of SmMef2,133. Nonetheless, these data, and recently published data in mice, corroborate that Mef2 plays a role in regulating the WNT pathway, a connection that has not been extensively explored.

In Drosophila and in mammals, Mef2 activates genes that participate in the Notch-Delta, Hedgehog, Fibroblast Growth Factor and Epidermal Growth Factor pathways [41]. This report adds the WNT pathway to that list (Figure 7). It will be of interest to further examine the role of SmMef2 in these pathways in schistosomes.

The use of PEI for transfection is a simple tool that can be used to dissect schistosome genetic pathways. We have not yet tested whether it facilitate the transfection of nucleic acids into other stages of schistosomiasis, but not necessarily into mammalian cells in culture, for whole snails, and for schistosomes. Whether it facilitate the transfection of nucleic acids into other stages of schistosomiasis, nor have we made a direct comparison between PEI transfection and electroporation, which could be informative. The successful use of PEI for the transfection of mammalian cells in culture, for whole snails, and for schistosomula, provides promise that this approach may work in other schistosome stages, and that it could be successfully used for other flat or roundworm species that have been challenging to transfct.

In addition, we are currently evaluating commercial and noncommercial promoters for their ability to drive gene expression in schistosomes, using PEI for nucleic acid delivery. Eventually, we expect that schistosome expression vectors could be selected based on promoter transcription rates, stage-specific or location dependent expression, or for use as cellular markers. In addition, since PEI is thought to function by protecting nucleic acid from digestion [35], RNA interference constructs may be potentially used for transcript knockdown by using this approach.

Supporting Information

Figure S1 cDNA quality test by amplification of Sm23 gene fragment. A 374 bp Sm23 gene fragment was PCR amplified from 20 ng of cDNA of each sample. Lane 1–4 from left to right are: 1. 1 Kb Plus DNA Ladder. 2. Sample treated with PEI and pEJ1181. 3. Sample treated with PEI and a mixture of pEJ1116. 4. Sample treated with PEI and pEJ1175. 5. Sample treated with PEI alone. 6. Sample treated with pEJ1181 alone. 7. Blank control treated without both PEI and vector DNA, respectively.

Table S1 Gene names and primer sequences used for quantitative PCR analysis. Gene names and DNA oligonucleotide sequences used for qRT-PCR analysis

Table S2 PEI does not deleteriously affect schistosome survival under conditions used for transfection. Survival rate of schistosomes was assayed over a two-day period in the presence or absence of PEI in RPMI complete media. Viable schistosome number was quantified at 1 hour, 1 day, and 2 days.

Table S3 The potential downstream targets of SmMef2 picked for expression test. Potential targets of SmMef2 tested for transcript level variations after overexpression of SmMef2,133

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Author Contributions

Conceived and designed the experiments: ERJ SL. Performed the experiments: SL ERJ. Analyzed the data: ERJ SL. Contributed reagents/materials/analysis tools: ERJ SL MK. Wrote the paper: ERJ SL.
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