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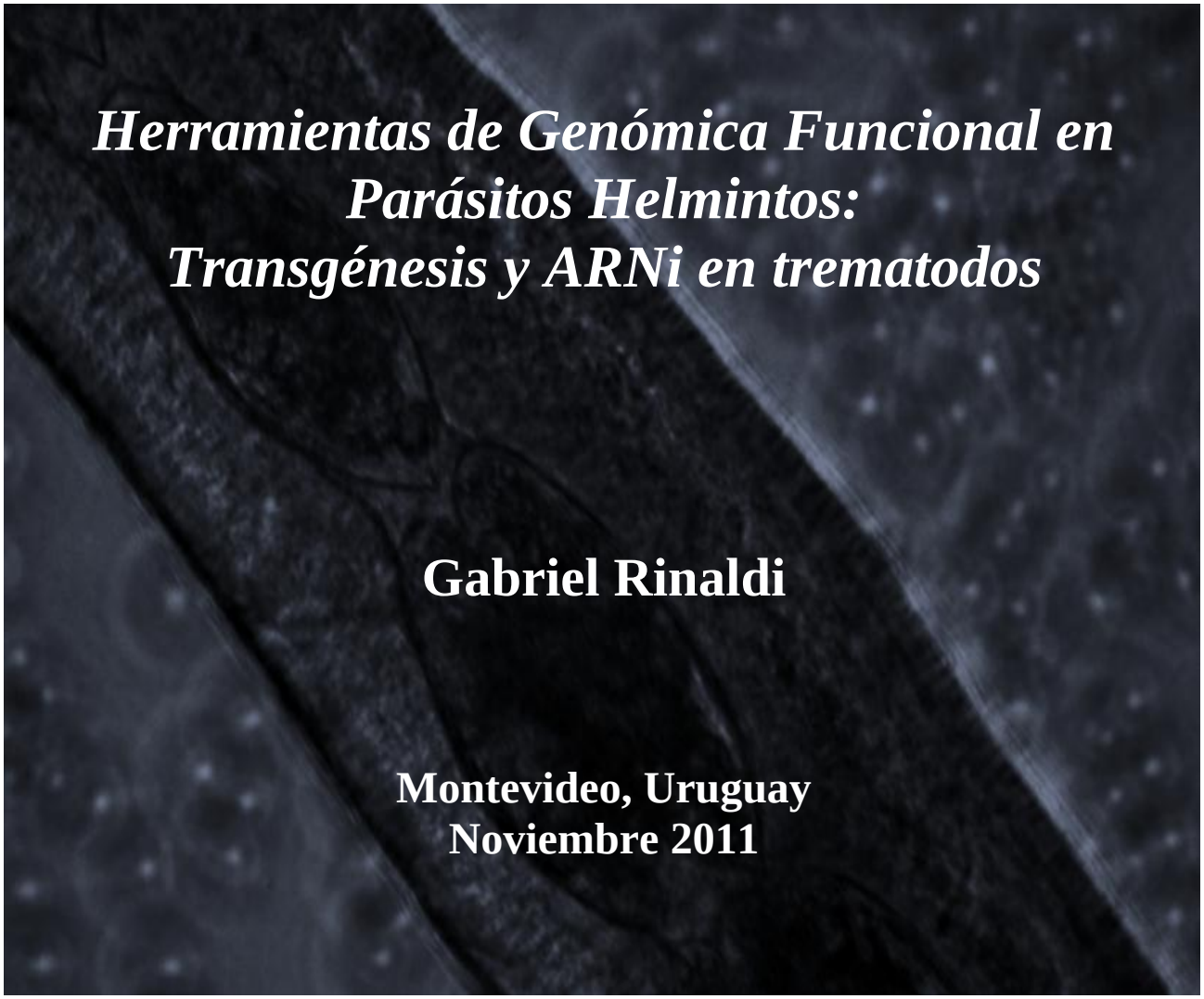

Herramientas de Genómica Funcional en Parásitos Helmintos: Transgénesis y ARNi en trematodos

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"…Y entonces, un día, llegó una criatura cuyo material genético no era muy diferente de las estructuras moleculares reproductoras de cualquier otra clase de organismos del planeta, que dicha criatura llamó Tierra. Pero era capaz de reflexionar sobre el misterio de su origen, de estudiar el extraño y tortuoso sendero por el cual había surgido desde la materia estelar. Era el material del Cosmos contemplándose a sí mismo. Consideró la enigmática y problemática cuestión de su futuro. Se llamó a si mismo humano. Y ansió regresar a las estrellas"

Carl Sagan (1934-1996)

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ABREVIATURAS

ADNc	ADN copia
ADNp	AND plasmídico
ARN pol	ARN polimerasa
ARNi	ARN de interferencia
ARNdc	ARN de doble cadena
cHS4	"chicken hypersensitive site 4" (aislante de cromatina)
kD	kiloDalton
miRNA	microRNA
MLV	virus de la leucemia murina modificado
neoR	gen de resistencia a la neomicina
pb	pares de bases
PBS	Tampon Fosfato Salino
PCR	Reacción en cadena de la polimerasa
qPCR	Reacción en cadena de la polimerasa cuantitativa
RAP	"Retrotransposon Anchored PCR"
qRAP	"quantitative RAP"
PM	Peso Molecular
RISC	"RNA-induced silencig complex"
siRNA	"short-interfering RNA"
shRNA	"short-hairpin RNA"
VSVG	"vesicular stomatitis virus glycoprotein"

Contenido

RESUMEN

Las infecciones parasitarias producidas por helmintos determinan un impacto global en salud pública que excede al producido por infecciones más estudiadas como malaria y tuberculosis. Es de primordial interés comprender su biología para generar nuevas y más eficientes herramientas diagnósticas, pronósticas y terapéuticas que redundarán en mejores programas de intervención y control parasitario. En particular, la schistosomiasis producida por platelmintos trematodes del género *Schistosoma* es considerada una de las helmintiasis más importantes en términos de morbilidad y mortalidad afectando a más de 200 millones de personas a nivel mundial y más de 800 millones en riesgo de infección.

La asignación de roles funcionales a las secuencias que se van generando de los proyectos genoma y transcriptoma representa un requisito primordial que se ha visto postergado en modelos parasitarios debido a la ausencia de herramientas de genómica funcional. En este sentido, desarrollar y optimizar herramientas de estudio de función génica como la transgénesis y la interferencia de ARN en schistosomas y otros parásitos trematodos, permitirá encontrar blancos moleculares potenciales de nuevas drogas y vacunas.

Considerando las particularidades del ciclo de vida complejo de los schistosomas, y bajo la hipótesis de que es posible (1) el desarrollo de la transgénesis y (2) la optimización de la interferencia de ARN convencional (mediada por ARN doble cadena) y no convencional (mediada por vectores codificantes de pequeños ARNs horquilla o shRNA – del inglés ‘small hairpin RNA’) en *Schistosoma mansoni*, nos propusimos los siguientes objetivos:

INTRODUCCIÓN

1. Los parásitos helmintos

1.1. Parasitosis desatendidas

Las infecciones parasitarias producidas por helmintos determinan un impacto global en salud pública que excede al producido por infecciones más estudiadas como malaria y tuberculosis (Hotez *et al.* 2008). En particular la schistosomiasis producida por platelmintos trematodes del género *Schistosoma* es considerada la helmintiasis más importante en términos de morbilidad y mortalidad afectando a más de 200 millones de personas a nivel mundial y más de 800 millones en riesgo de infección (Hotez *et al.* 2008; Brindley *et al.* 2009). En África sub-sahariana las helmintiasis son co-endémicas con la malaria, el HIV/SIDA y la tuberculosis, lo que agrava de por si la presentación clínica y la morbimortalidad de estas afecciones (Hotez *et al.* 2008).

Por su parte las trematodiasis hepática (producida por gusanos de las especies *Clonorchis sinensis, Fasciola gigantica, Fasciola hepatica, Opisthorchis felineus* y *Opisthorchis viverrini*), pulmonar (*Paragonimus* spp), e intestinal (*Echinostoma spp.* y *Fasciolopsis buski*) afectan a otros 100 millones de personas fundamentalmente en las áreas más pobres del planeta, China, India, sudeste asiático, altiplano sudamericano y Africa sub-sahariana (Keiser and Utzinger 2009). A esto se le suma la asociación entre las infecciones producidas por algunos trematodes como *Schistosoma haematobium*, *Clonorchis sinensis* y *Opistorchis viverrini* con la carcinogénesis. Dichos parásitos son considerados carcinogénicos del grupo 1 por la Organización Mundial de la Salud (OMS) (Vennervald and Polman 2009), aunque los mecanismos moleculares y/o celulares involucrados son aún desconocidos (Botelho *et al.* 2009).

Al gran impacto en la salud humana en términos de morbi-mortalidad hay que sumarle el efecto negativo producido por las helmintiasis en especies animales productoras, originando pérdidas directas por disminución de peso, calidad de carne, lana o leche, o indirectas por los costos de tratamiento o pérdida de la fuerza de trabajo en aquellos países menos industrializados donde se usa tracción animal. Las pérdidas

económicas causadas por dicha parasitosis en rumiantes domésticos a nivel mundial alcanzan los 2000 millones de dólares anuales afectando fundamentalmente aquellos países cuyas economías dependen en gran parte de la producción agrícola ganadera (Spithill and Dalton 1998).

La naturaleza emergente de alguna de estas parasitosis no solo en los países más pobres (McCarthy and Moore 2000; Keiser and Utzinger 2005; Hotez *et al.* 2009), sino también en países industrializados (Fried and Abruzzi 2010) aumenta aún más su impacto a nivel médico y veterinario. Sin embargo, estas enfermedades han sido subestimadas y relegadas en cuanto al apoyo financiero que han recibido los programas para su estudio y evaluación, integrando en su conjunto las llamadas "enfermedades tropicales desatendidas" (Engels and Savioli 2006; Stein *et al.* 2007). El control de estas parasitosis es complejo, pues si bien existen drogas que son efectivas, estas son costosas, pueden causar tolerancia y hepato-toxicidad, y en general no impiden la re-infección. Existe también preocupación en cuanto a la aparición de resistencia contra las drogas más frecuentemente utilizadas como los benzimidazoles (albendazol, praziquantel, triclabendazol) (Brennan *et al.* 2007; Abdul-Ghani *et al.* 2009; Couto *et al.* 2011). Por todo esto existe una creciente necesidad de desarrollar nuevas estrategias de control parasitario, técnicas de diagnóstico más eficientes y en particular identificar blancos potenciales para el diseño de nuevas drogas y/o vacunas.

1.2. Schistosomas como modelo de trematodos

Dentro de los parásitos trematodos se han tomado las especies del género *Schistosoma* como modelos de estudio debido a su gran relevancia sanitaria. Theodor Bilharz en 1852 describió por primera vez la Schistosomiasis como la enfermedad tropical causada por parásitos trematodos del genero *Schistosoma*, también llamada "bilharzia". Estos parásitos infectan a más de 200 millones de personas en el planeta, con más de 800 millones en riesgo de infección, fundamentalmente distribuidas en regiones tropicales del globo como zonas costeras de Brasil, Venezuela e islas del caribe, África sub-sahariana, algunos regiones aisladas en medio oriente, y el sudeste asiático

(Figura 1) (Gryseels *et al.* 2006; Hotez *et al.* 2008). Solo en África se calcula que la Schistosomiasis causa aproximadamente 300 000 muertes por año (Pearce and Freitas 2008).

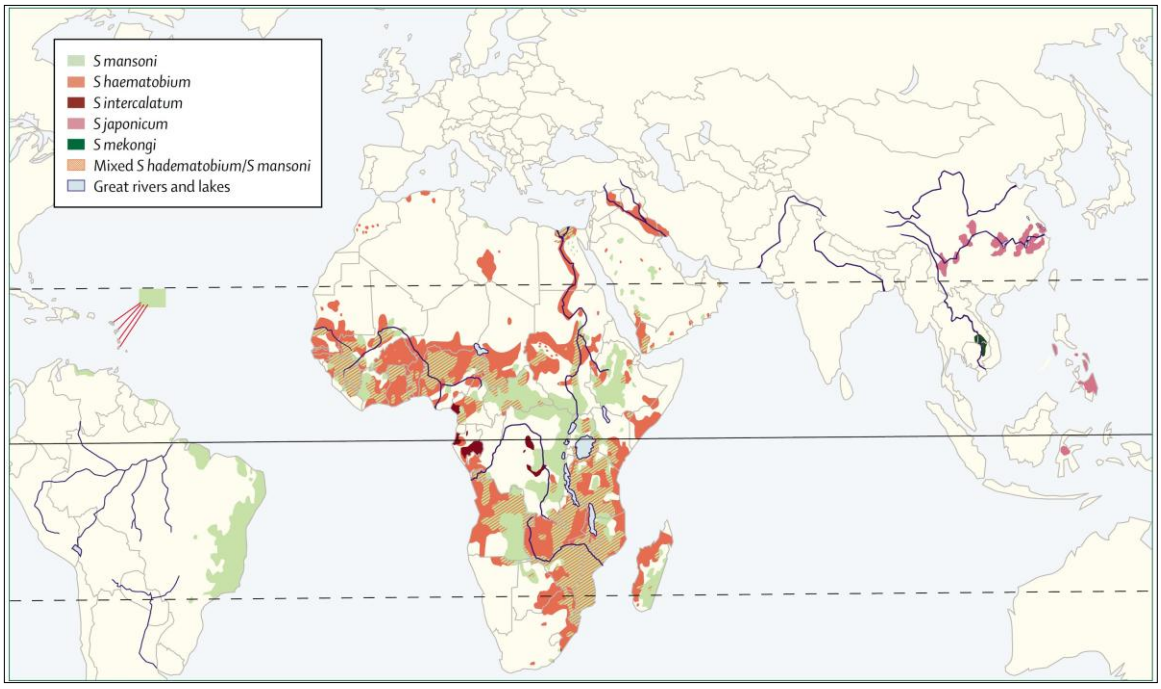

Figura 1. Distribución global de la schistosomiasis. *Principales áreas de infección endémica: Schistosoma mansoni- África sub-sahariana, nordeste de Brasil, Surinam, Venezuela, islas del caribe, bajo Egipto y península arábiga; Schistosoma haematobium- África sub-sahariana, valle del Nilo en Egipto y Sudán, medio oriente y península arábiga; Schistosoma japonicum- a lo largo de los lagos centrales y el río Yangtze en China, islas de las Filipinas y pequeñas regiones en Indonesia; Schistosoma mekongi- Laos, Camboya y Tailandia; Schistosoma intercalatum- algunas regiones de África central y occidental. Extraído y adaptado de (Gryseels et al. 2006).*

Schistosoma es un trematodo digenético, perteneciente a la superfamilia Schistosomatoidea. Al igual que el resto de los trematodos, los schistosomas presentan un ciclo de vida complejo que involucra dos huéspedes, un molusco como huésped intermediario y a un mamífero (fundamentalmente el ser humano) como huésped definitivo. Sin embargo solo esta familia de trematodos presenta dimorfismo sexual con gusanos machos y hembras bien diferenciados en el estadio adulto. La hembra y el macho apareados, habitan en las venas mesentéricas del huésped definitivo, donde se reproducen sexuadamente con la correspondiente producción de huevos fértiles, mientras que en el

huésped intermediario el parásito se reproduce asexuadamente determinando una "expansión clonal" de los estadios intracaracol.

Cada especie tiene un tropismo particular en el huésped definitivo, que determina diferencias en la patogenia y sintomatología de cada infección. Las tres especies más importantes responsables de la schistosomiasis en humanos son *Schistosoma mansoni* y *Schistosoma japonicum*, responsables de schistosomiasis intestinal y hepática, y *Schistosoma haematobium*, responsable de la schistosomiasis urinaria. Otras dos especies de schistosomas de importancia menor que infectan al ser humano son *S. intercalatum* y *S. mekongi*. Los especímenes adultos de *S. mansoni* residen en las vénulas de los plexos mesentéricos superiores, y los de *S. japonicum* residen en las vénulas de los plexos mesentéricos inferiores, y venas hemorroidales superiores. *S. haematobium* reside en los plexos venosos vesicales y ureterales inferiores, causando sintomatología a nivel urinario fundamentalmente.

Los huevos liberados por las hembras penetran las paredes de los vasos sanguíneos y generan su propio trayecto a través de la mucosa del tejido infectado hasta ser liberados con las heces al exterior. Muchos de ellos quedan retenidos en el hígado generando fibrosis y granulomas característicos responsables de la patogenia de esta infección en el caso de la schistosomiasis hepatica/intestinal (*S. mansoni* y *S. japonicum*). Los huevos liberados por las hembras de *S. haematobium* quedan retenidos en la mucosa del tracto genitourinario siendo responsable de las manifestaciones clínicas de la schistosomiasis haematobia.

Una vez que los huevos alcanzan agua fresca, eclosionan y liberan una larva ciliada, miracidio, que nada e infecta al huésped intermediario. *S. mansoni* utiliza para propagarse fundamental caracoles del genero *Biomphalaria, S. japonicum,* caracoles del genero *Oncomelania*, y *S. haematobium,* caracoles del genero *Bulinus*. De esta manera, la distribución geográfica de las diferentes especies de schistosomas depende esencialmente de la ecología de sus respectivos huéspedes intermedios.

El miracidio penetra el caracol, pierde las cilias y se transforma en esporocisto madre. En el interior del esporocisto se encuentran masas de células germinales e indiferenciadas que dividiéndose en forma asexuada producirá esporocistos hijas que migran al tracto digestivo del caracol comenzando a generar cercarias. Miles de cercarias, clonales entre sí pueden derivar de un solo esporocisto hija. Este proceso lleva entre 4 y 5 semanas, luego del cual las cercarias comienzan a liberarse, y en pocas horas encuentran e infectan al huésped definitivo. La cercaria penetra la piel del huésped definitivo, pierde la cola y se transforma en schistosomula que a través de la circulación linfática, pasa por los pulmones y el corazón hasta llegar a su lugar definitivo, las vénulas mesentéricas a nivel del intestino. Aquí se desarrollan a adultos, se aparean machos y hembras, y comienza la producción y liberación de huevos al exterior, cerrando el ciclo (**Figura 2**).

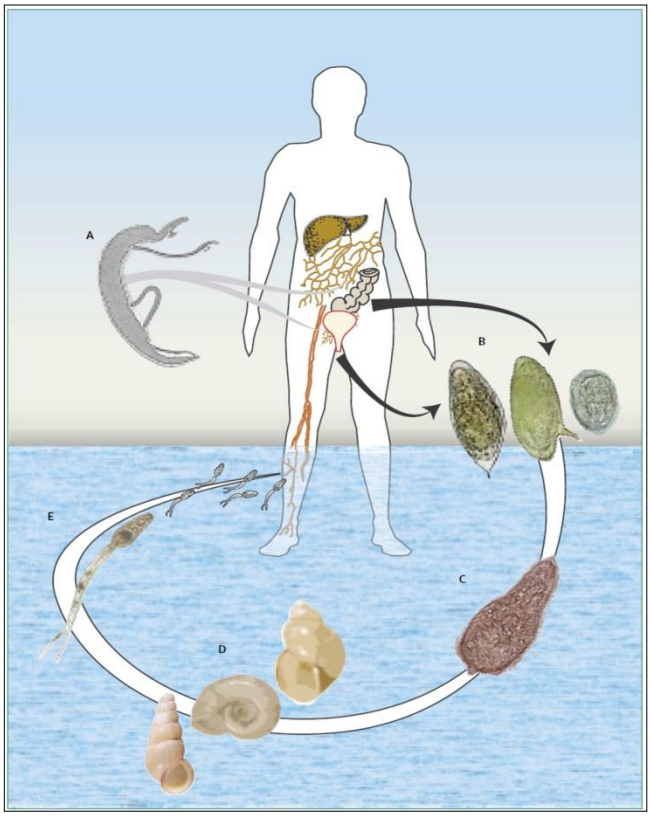

Figura 2. Ciclo de vida de los Schistosomas. *A. Adultos apareados, B. huevos, de izquierda a derecha de S. haematobium, S.mansoni y Sjaponicum, C. miracidio, D. huesped intermediario, caracoles infectados por las distintas especies de Schistosoma, E. cercarias, estadio invasivo que penetra la piel del huesped definitivo. Adaptado de (Gryssels 2006).*

La schistosomiasis puede presentarse en forma aguda, con manifestaciones clínicas básicamente vinculadas a la penetración de la larva cercaria por la piel y la dispersión por el sistema linfático y/o circulatorio de las schistosomulas. La severidad de la sintomatología asociada a esta primera etapa de infección con el parásito depende en gran medida de la carga parasitaria y del sistema inmune del huésped. En casos severos la schistosomiasis aguda, también denominada fiebre de Katayama, se manifiesta a las pocas semanas luego de la exposición del individuo a las cercarias como una reacción sistémica de hipersensibilidad a la parasitemia. Se presenta como un cuadro de sintomatología general inespecífica dada por fiebre, artromialgias, dolor abdominal difuso, síndrome de repercusión general que suele revertir espontáneamente al cabo de unos 2 a 5 días.

Sin tratamiento específico los parásitos se establecen en el huésped generando la patología crónica dada por la formación de granulomas y fibrosis, debido fundamentalmente al depósito de los huevos en la mucosa intestinal y hepática. Dicha fibrosis conduce progresivamente al deterioro de la función hepática, hipertensión portal, ascitis y eventualmente a la muerte del paciente por hemorragia severa a partir de varices esofágicas. El grado de severidad de las lesiones a nivel hepático (*S. mansoni y S. japonicum*) y urinario (*S. haematobium*) depende no solo de la carga de huevos retenidos sino también de la respuesta inmune del huésped. Manifestaciones generales como eosinofilia, anemia, y reacciones de tipo inmunoalérgico suelen acompañar a la infección crónica. Trabajos recientes muestran que el número de casos de schistosomiasis a nivel mundial es ampliamente subestimado debido a las presentaciones atípicas y estados subclínicos de la enfermedad. Síntomas aislados como anemia, dolor abdominal crónico, diarrea, intolerancia al ejercicio, bajo crecimiento y alteraciones en el desarrollo normal del niño pueden ser la manifestación de una infección con este trematodo (Gryseels *et al.* 2006). Técnicas clásicas como el hallazgo de huevos característicos de cada especie en un coproparasitario, y en muestras de orina siguen siendo las herramientas diagnosticas más utilizadas (**Figura 3**). La serología, técnicas imagenológicas y endoscópicas colaboran para realizar un diagnóstico más preciso.

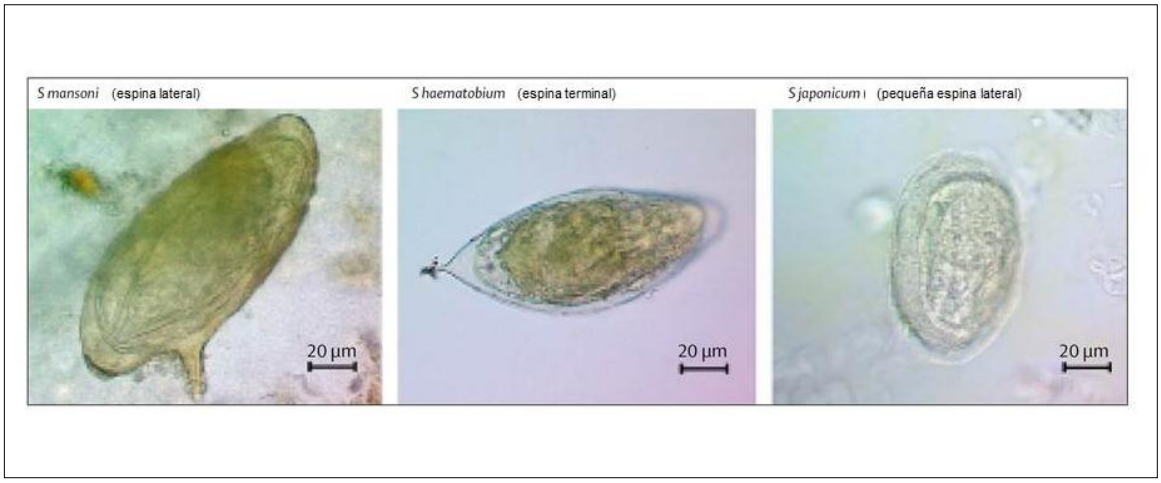

Figura 3. Huevos de las tres especies más importantes de Schistosoma*. De izquierda a derecha: S. mansoni, S. haematobium, S. japonicum. Extraído y adaptado de (Gryseels et al. 2006).*

La droga más efectiva para el tratamiento de la schistosomiasis es el praziquantel, pero la preocupación por la aparición de reportes de resistencia a la misma, y la necesidad de encontrar nuevas opciones terapéuticas obligan a la comunidad científica a comprender mejor los mecanismos de interacción huésped-parásito para encontrar blancos de drogas alternativas y/o desarrollo de vacunas (Gryseels *et al.* 2006; Hotez *et al.* 2008; McManus 2008; Couto *et al.* 2011).

1.3. Asociación de la schistosomiasis con otras patologías

Se han demostrado vínculos patogénicos entre algunas infecciones crónicas producidas por virus, bacterias, y algunos parásitos helmintos, y la carcinogénesis. Casi un 20% de las neoplasias podrían estar asociadas a un agente infeccioso en particular. Asimismo se ha calculado que la incidencia de cáncer podría disminuir un 26% en algunos países donde el agente infeccioso causal es endémico si estas infecciones se tratan y previenen adecuadamente (Vennervald and Polman 2009). Aunque solo una fracción menor de los cánceres vinculados a agentes infecciosos es atribuida a parásitos helmintos, la elevada prevalencia de alguna de estas parasitosis en regiones endémicas

del globo hace que existan poblaciones humanas con riesgo elevado a desarrollar estas neoplasias. El estado de inflamación crónica producida por la infección reiterada de estos helmintos en estas poblaciones de riesgo determina la generación de condiciones carcinogénicas en los tejidos primariamente afectados. Aunque los mecanismos moleculares y/o celulares involucrados en la carcinogénesis mediada por la infección de dichos parásitos son aún desconocidos, varios grupos están generando resultados promisorios en esta línea (Botelho *et al.* 2009; Young *et al.* 2010).

Los parásitos trematodos *Schistosoma haematobium*, *Clonorchis sinensis* y *Opistorchis viverrini* actualmente integran el grupo 1 de carcinogénicos por la Organización Mundial de la Salud (OMS) (Parkin 2006; Bouvard *et al.* 2009; Vennervald and Polman 2009). En particular la incidencia de *S. haematobium* en todo el continente africano y algunas regiones de medio oriente hace que la incidencia de cáncer de vejiga asociado a esta parasitosis sea extremadamente elevada en estas regiones. Existe más gente está infectada con *S. haematobium* que con los otros schistosomas combinados, especialmente en regiones co-endémicas con otras helmintiasis, malaria, tuberculosis y HIV/SIDA. De los más de 110 millones de casos de schistosomiasis haematobia en Africa sub-sahariana, 70 millones están asociados con hematuria, 18 millones con patología mayor de las vías urinarias, y 10 millones con hidronefrosis que puede determinar una enfermedad renal severa(van der Werf *et al.* 2003; King *et al.* 2005; Hotez *et al.* 2009). En muchos pacientes la inflamación crónica en respuesta a los huevos depositados en la mucosa del órgano conduce a carcinoma escamoso de vejiga (Hodder *et al.* 2000; Shiff *et al.* 2010).

No se ha encontrado una asociación directa entre la infección con *S. haematobium* y cáncer de cuello de útero. Sin embargo, las lesiones producidas en la mucosa cervico-vaginal por el parásito pueden incrementar las probabilidades de contagio del virus del papiloma humano (HPV), patógeno que si se vincula con la génesis de neoplasia cervical (Vennervald and Polman 2009).

Entre un 25% y 75% de las mujeres infectadas con *S. haematobium* sufren de schistosomiasis genital femenina (SGF) del tracto genital inferior (Hotez *et al.* 2009). La SGF resulta de la deposición de los huevos del parásito en la mucosa del útero, cérvix, vagina y/o vulva, determinando una respuesta inflamatoria por parte del huésped que conduce a la formación de granulomas, fibrosis y alteración de la vascularización (Kjetland *et al.* 2005).

Por otro lado, se ha demostrado una relación entre la SGF y el aumento de la susceptibilidad al HIV/SIDA, probablemente vinculado a las lesiones localizadas en el tracto genital, inflamación y aumento de la vascularización (Feldmeier *et al.* 1994; Kjetland *et al.* 2006; Ndhlovu *et al.* 2007). En la **figura 4** se observa la superposición de las áreas de mayor prevalencia de schistosomiasis haematobia y HIV/SIDA (Hotez *et al.* 2009). La disminución en la fertilidad femenina debido a complicaciones de la schistosomiasis haematobia también ha sido reportada recientemente (Kjetland *et al.* 2010). Por lo anterior, los schistosomas y en particular *S. haematobium* se transforman en excelentes modelo de estudio de factores de aumento de susceptibilidad al cáncer, a enfermedades de transmisión sexual, (en particular HIV/SIDA) e infertilidad femenina.

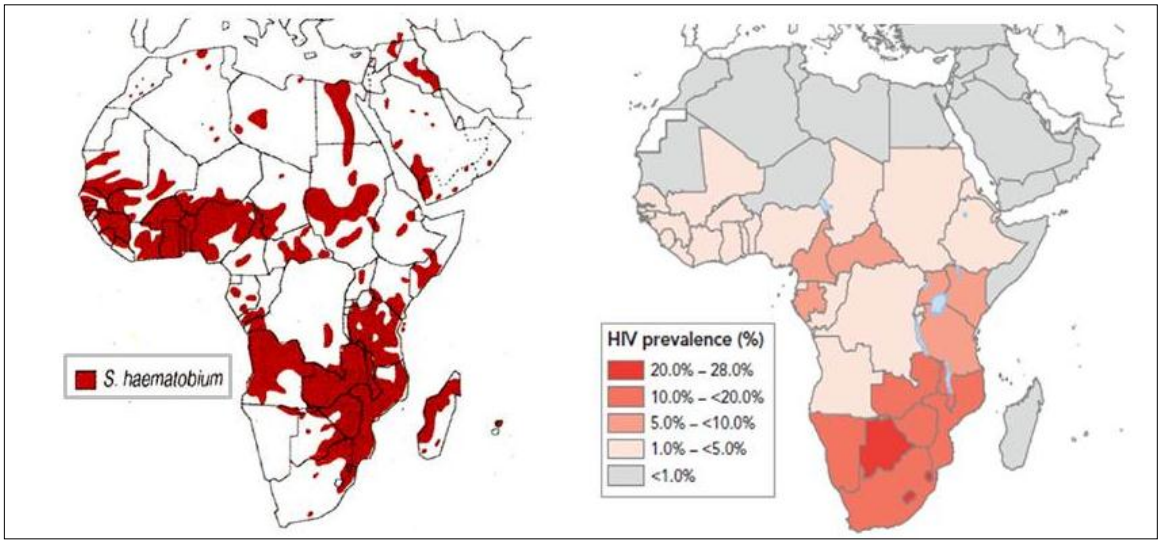

Figura 4. Distribución global de Schistosoma haematobium (izquierda) y de HIV/SIDA (derecha) en África. *Adaptado de (Hotez et al. 2009).*

2. Genómica de los parásitos helmintos

2.1. Contribución de la Genómica a la Parasitología

La parasitología molecular surge como una disciplina que permitirá avanzar en el estudio de la biología básica de las parasitosis desatendidas contribuyendo al desarrollo de nuevas herramientas diagnósticas y terapéuticas. Hemos comenzado a transitar la "era post genómica" de la parasitología molecular marcada por el acelerado avance de los proyectos de secuenciación global de genomas de parásitos helmintos de importancia médica, veterinaria y agropecuaria (Brindley *et al.* 2009; Chuan *et al.* 2010). La **tabla 1** extraída de Brindley *et al.*, 2009 agrupa los proyectos de secuenciación genómica de varios parásitos helmintos en progreso o ya concluidos. Grandes avances se han realizado en genomas de parásitos nematodes (http://www.nematode.net/), incluyendo los parásitos *Necator americanus* y *Ancylostoma caninum* (Ababucker *et al.* 2008). Asimismo la secuencia completa del genoma del parásito *Bruglia malayi* responsable de la filariasis fue recientemente reportada (Ghedin *et al.* 2007) y el proyecto genoma de *Onchocerca volvulus* está en progreso (**Tabla 1**).

En los platelmintos los primeros esfuerzos se centraron en la catalogación de los genes expresados por un parásito en determinado estadio, condición o tejido (transcriptoma). De esta forma se conocen datos del transcriptoma de varias especies de tremátodos: *S.mansoni* (Verjovski-Almeida *et al.* 2003), *S.japonicum* (Hu *et al.* 2003), *Opistorchis viverrini* y *Clonorchis sinensis* (Laha *et al.* 2007; Young *et al.* 2010), *Paragonimus westermani* (Kim *et al.* 2006), *Echinostoma paraensei* (Adema *et al.* 2000), *F. hepatica* (Cancela *et al.* 2010; Young *et al.* 2010) y los céstodos *Moniezia expansa* (Zhao *et al.* 2009), *E. granulosus* (Fernandez *et al.* 2002) y *T. solium* (Almeida *et al.* 2009).

Table 1. Human parasitic helminths (and their close relatives) with genome sequencing projects completed or underway.

Phylum or Class	Species	Common Name / Disease	Primary host	Genome size, Mb	GenBank Project ID	cDNAs (3730 ABI), 1,000 s	Genome Sequencing Status	Sequencing Institute[a]
Nematoda (roundworms)								
Clade V[b]	*Necator americanus*	Hookworm/necatoriasis	Human	—	20369	5	In progress	WUGC
	Ancylostoma caninum	Model hookworm	Dog	344	12841	81	Improving draft	WUGC
	Nippostrongylus brasiliensis	Model hookworm	Rat	—	20445	14.7	In progress	SI
Clade IV	*Strongyloides stercoralis*	Threadworm/strongyloidiasis	Human	—	—	11.4	In progress	SI
	S. ratti	Model threadworm	Rat	—	—	27.4	In progress	SI/WUGC
Clade III	*Ascaris lumbricoides*	Large roundworm/ascariasis	Human	230	—	1.8	In progress	SI
	A. sum	Model large roundworm	Pig	230	—	55.7	Improving draft	WUGC/SI
	Brugia malayi	Filaria/lymphatic filariasis	Human	96	9549	26.2	Improving draft	TIGR/University of Pittsburgh
	Loa Loa	Filaria/loaisis (cutaneous filariasis)/African eye worm	Human	—	—	3.3	In progress	BI
	Onchocerca volvulus	Filaria/river blindness	Human	150	—	15	In progress	SI
	Acanthocheilonema viteae	Model filaria	Rodent	—	33239	0	In progress	UMIGS
Clade I	*Trichinella spiralis*	Trichina worm/trichinosis	Pig to human	71	12605	253	Draft completed	WUGC
	Trichuris trichiura	Whipworm/trichuriasis	Human	—	—	0	In progress	SI
	T. muris	Model whipworm	Mouse	96	—	7	In progress	SI
	T. suis	Model whipworm	Pig	-	—	0	In progress	WUGC
Cestoda (tapeworms)	*Echinococcus multilocularis*	Tapeworm/alveolar hydatidosis	Rodent; larva infects humans	150	—	1	In progress	SI
	E. granulosus	Tapeworm/unilocular hydatidosis	Canids; larva infects humans	150	12620	10	In progress	SI
	Taenia solium	Pork tapeworm/taeniasis/cysticercosis	Human	270	17815	25	Draft completed	Mexico City
Trematoda (flukes)	*Schistosoma mansoni*	Blood fluke/intestinal schistosomiasis	Human	390	12599	206	Draft completed	SI/TIGR
	S. haematobium	Blood fluke/urinary schistosomiasis	Human	—	12616	0	In progress	SI
	S. japonicum	Blood fluke/intestinal schistosomiasis	Human	400	29491	104	Draft completed	CNHGC
	Clonorchis sinensis	Liver fluke/clonorchiasis	Human	—	17975	3	In progress	SNUCM

[a]WUGC, Washington University's Genome Center.
[b]Phylogeny based on Blaxter et al. [47].
BI, Broad Institute; CNHGC, Chinese National HGC; SI, Sanger Institute; SNUCM, Seoul National University College of Medicine; TIGR, The Institute for Genomic Research (now JCVI).

Tabla 1 *Parásitos helmintos con proyecto genoma complete o en curso. Extraído y adaptado de (Brindley et al. 2009)***.**

Se han publicado recientemente y están disponibles los genomas completos de *Schistosoma mansoni* (http://www.sanger.ac.uk/Projects/S_mansoni/) y *S. japonicum* (http://function.chgc.sh.cn/sj-proteome/index.htm), dos de las tres especies de schistosomas más prevalentes junto a *S. haematobium* (Berriman *et al.* 2009; Brindley *et al.* 2009; Liu *et al.* 2009). Estos son los primeros genomas secuenciados de platelmintos, y más aún, los primeros genomas secuenciados de organismos pertenecientes al linaje de los lofotrocozoos, lo que agrega un gran interés desde el punto de vista evolutivo.

Clásicamente se ha considerado a los platelmintos como los primeros organismos con simetría bilateral. Este modelo de evolución de los metazoarios se basa en varios aspectos: son organismos acelomados, no segmentados, con tubo digestivo incompleto (incluso ausente en cestodos), hermafroditas (salvo en el caso de los *Schistosomas*) y con protonefridios como órganos excretores. Recientemente la inclusión de datos moleculares ha permitido modificar notablemente este modelo filogenético. A los organismos protostomados se los divide en dos linajes principales, los lofotrocozoos y los ecdisozoos. El primer grupo, extremadamente diverso, lo constituyen los platelmintos, los anélidos y los moluscos mientras que el segundo linaje está formado por organismos con cutícula externa que mudan periódicamente como los nemátodos, artrópodos y algunas phyla menores (**Figura 5**). Consecuentemente los organismos bilaterales derivados de un ancestro en común con los cnidarios hace unos 630 millones de años se agruparían en tres linajes básicos: (1) lofotrocozoos y (2) ecdisozoos (ambos protostomados) y (3) deuterostomados (equinodermos, cordados, vertebrados) (Baguna and Riutort 2004; Lartillot and Philippe 2008).

Los genomas de los schistosomas se caracterizan por la presencia de gran número de secuencias repetidas, derivadas en su mayoría por elementos transponibles (retrotransposones y transposones) y una estructura inusual con intrones pequeños en la porción 5' y grandes en la región 3' terminal. Se desconoce la razón de esta particularidad, así como si ésta es una característica propia de los *Schistosomas*, de los platelmintos o de todos los lofotrocozoos.

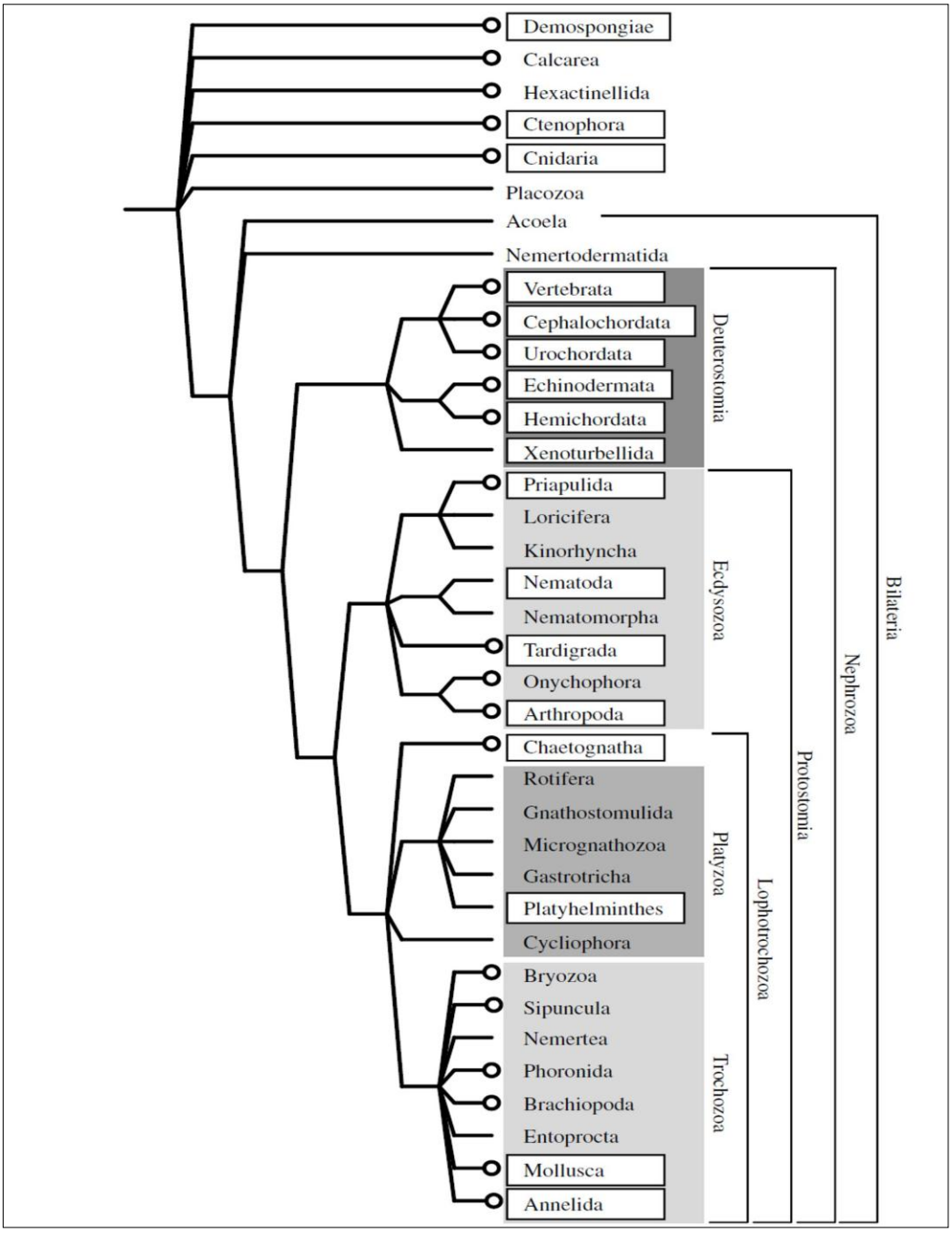

Figura 5. Árbol filogenético de los metazoarios basado en datos morfológicos y moleculares. *Los protostomados quedan divididos en dos grandes grupos, ecdisozoa y lofotrocozoa. Los phyla resaltados en un recuadro blanco son aquellos para los que se encuentran disponibles bases de datos de genoma o transcriptoma. Extraído y modificado de (Giribet 2008).*

Se estiman unos ~13000 genes codificantes de proteínas los cuales estarían sujetos a complejos mecanismos de regulación de expresión génica incluyendo splicing, splicing alternativo y transplicing (Verjovski-Almeida and DeMarco 2011), metilación del ADN (Geyer *et al.* 2011), pequeños ARNs regulatorios (Copeland *et al.* 2009; de Souza Gomes *et al.* 2011) y otros mecanismos de regulación epigenética. Se ha logrado asignar un posible rol funcional a aproximadamente un 50% de genes, pero las restantes secuencias génicas no tienen función conocida aún y/o similitud con genes encontrados en otros organismos. Determinar si estas secuencias génicas son relevantes en le biología del parásito, fundamentalmente en su interacción con el hospedero, es un paso fundamental.

El acceso a nuevas secuencias génicas producto de los proyectos genomas ya concluidos y en progreso, así como la comprensión de los complejos mecanismos de regulación postranscripcional, de los transcriptomas y proteomas de *Schistosoma* permitirá encontrar posibles candidatos para el desarrollo de nuevas drogas y vacunas parasitarias (Chuan *et al.* 2010; Loukas *et al.* 2011; Verjovski-Almeida and DeMarco 2011). Para esto se hace necesario e imprescindible el desarrollo de herramientas de genómica funcional como la interferencia de ARN y la transgénesis que permitan identificar y comprender la función de las secuencias génicas presentes en el genoma parasitario (Beckmann and Grevelding 2011; Lok 2011). Asimismo, esto permitirá la validación del rol funcional de mediadores moleculares de la interfase huésped-parásito protagónicos en procesos vitales como la invasión, migración por diferentes tejidos, nutrición, evasión de la respuesta inmunitaria y establecimiento en los organismos hospederos. Comprender dichos mecanismos e identificar sus mediadores moleculares es de fundamental importancia no solo para la parasitología básica y sino también para la aplicada al desarrollo de nuevas estrategias de control parasitario (Tort *et al.* 1999; McKerrow *et al.* 2006; Rinaldi 2008).

3. Genómica funcional en *Schistosomas*

En las últimas décadas la Genómica Funcional ha avanzado en paralelo al desarrollo de herramientas de genética reversa que permitieron identificar funciones de secuencias génicas y vincularlas a un fenotipo particular. Es posible modificar directamente la secuencia nucleotídica de una proteína de función desconocida y analizar los fenotipos resultantes. Las aproximaciones fundamentales de la genética reversa han sido la mutagénesis dirigida (Sivasubbu *et al.* 2007), la transgénesis (Mann *et al.* 2008), la inactivación génica ("knock out") a través de su sustitución por una copia no funcional u otra secuencia (Koyanagi *et al.* 2008) y más recientemente las "tecnologías antisentido" e interferencia de ARN (Dallas and Vlassov 2006; Janitz 2007). La interferencia de ARN mediante ARN doble cadena (ARNi) surge como una estrategia alternativa que permite silenciar la actividad génica mediante la degradación especifica del ARNm, determinando la ausencia de la proteína y la posible generación de un fenotipo particular.

El análisis funcional de secuencias génicas en parásitos trematodos se ha visto relegado por la ausencia de herramientas de genética reversa y directa, así como de aproximaciones de ganancia y perdida de función. Esto se debe fundamentalmente a las particularidades de estos organismos grandes, complejos, multicelulares, con múltiples tejidos especializados, sin líneas celulares desarrolladas, y con diversos estadios de desarrollo adaptados a la vida libre o al parasitismo en dos huéspedes muy diferentes como lo son un molusco y un mamífero. Estas características hacen difícil mantener el ciclo de vida completo *in vitro*, un paso fundamental para el desarrollo de herramientas de manipulación génica en estos parásitos.

3.1. Schistosoma en el laboratorio: mantenimiento y cultivo de estadios

Aunque las tres especies principales de schistosomas se pueden mantener relativamente bien en cultivo mediante la infección *in vitro* de hospederos (la correspondiente especie de caracol como huésped intermediario y ratones y/o hámsteres como huéspedes definitivos), históricamente *S. mansoni* ha sido el más ampliamente estudiado (Lewis *et al.* 2008). Fundamentalmente esto es consecuencia de la relativa mayor facilidad de cultivo de esta especie con respecto a las otras dos. *S. japonicum* es la especie que genera mayor dificultad en el laboratorio, no solo por la dificultad de mantener en cultivo caracoles del genero *Oncomelania*, sino también porque esta especie determina una patología mucho más agresiva en el ratón. Solo uno o dos pares de parásitos adultos de *S. japonicum* son capaces de producir una severa enfermedad granulomatosa hepática, en contraste con la infección producida por *S. mansoni* donde más de 30 pares de parásitos adultos pueden ser recuperados de los ratones infectados que presentan severa patología hepática. De las tres especies de schistosomas de mayor relevancia en el ser humano, *S. haematobium* parece ser la más desatendida y son mucho menos los reportes científicos que refieren a esta especie. A pesar de que el mantenimiento en el laboratorio de los caracoles *Bulinus* es relativamente sencillo y similar a los caracoles del genero *Biomphalaria*, la principal causa de esta negligencia es la falta de un buen modelo biológico de huésped definitivo que reproduzca la patogenia de la schistosomiasis urinaria. Tanto en hámsteres, que es el modelo clásico utilizado para mantener el ciclo en el laboratorio, como en ratones, la forma de presentación de la enfermedad es intestinal y hepática con muy poca afectación urogenital (Lewis *et al.* 2008; Mann *et al.* 2010).

El mantenimiento en el laboratorio de los huéspedes intermediario y definitivo infectados con schistosomas permite acceder a diferentes estadios del desarrollo parasitario y cultivarlos por un tiempo bajo las condiciones adecuadas, posibilitando someterlos a diversos tratamientos (transfección de moléculas reporteras, ARNi, transgénesis, etc) y reintroducir eventualmente el parásito modificado al ciclo de vida mediante la infección del huésped correspondiente. Schistosomulas obtenidas mediante la

transformación *in vitro* de cercarias emitidas por caracoles infectados pueden ser cultivadas, transfectadas y reintegradas al ciclo de vida infectando de ratones mediante inyección intraperitoneal o intramuscular. De la misma forma los huevos pueden ser modificados genéticamente, eclosionados y los miracidios resultantes empleados para infectar caracoles. La **figura 6** muestra una representación esquemática de los estadios de desarrollo del schistosoma que pueden potencialmente ser sujetos a modificaciones génicas. Muchos de estos estadios pueden ser reintroducidos al ciclo de vida del parásito. La posibilidad de mantener en cultivo y modificar genéticamente a los schistosomas abrió las puertas al desarrollo de herramientas de genómica funcional en dicho parásito.

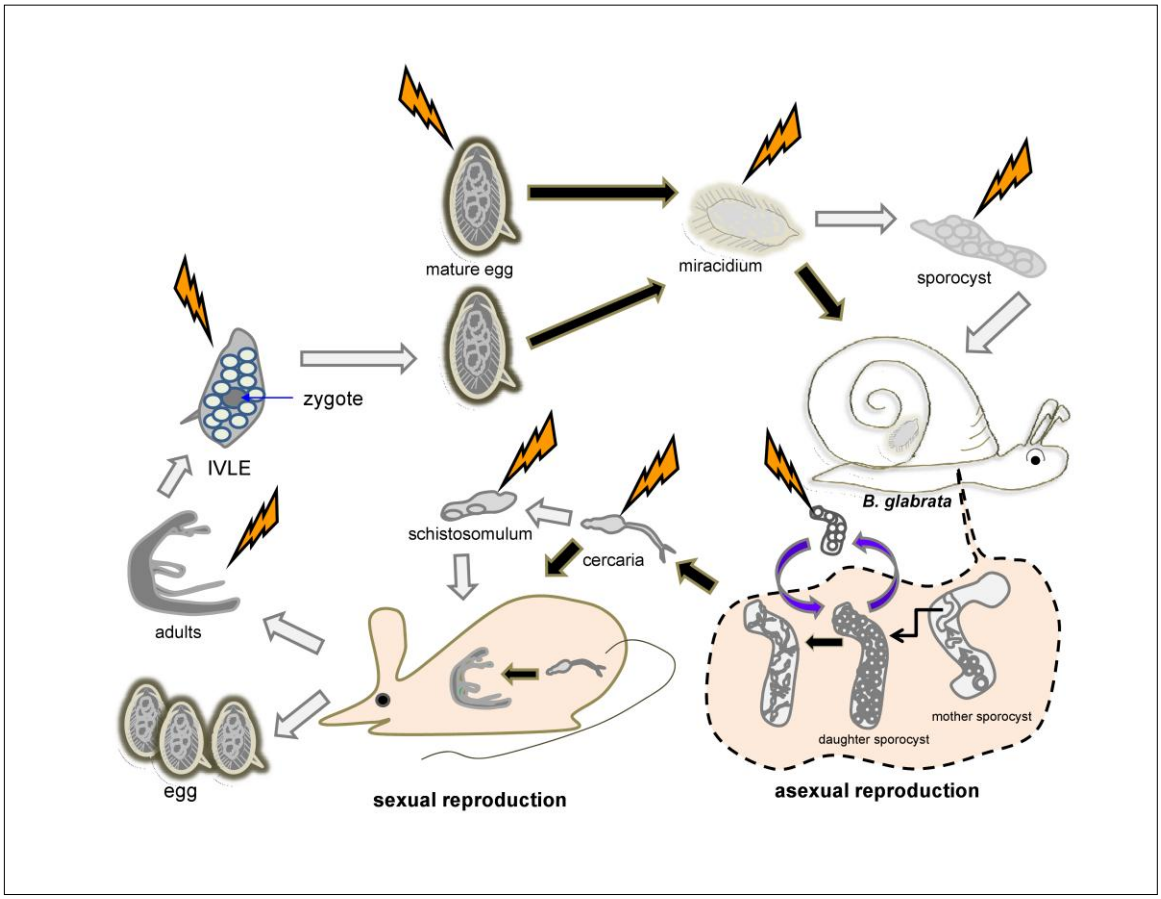

Figura 6. Representación esquemática mostrando los estadios del desarrollo del parásito que son susceptibles de manipulación génica. *Se muestran ambos hospederos, el mamífero vinculado a la reproducción sexual y el caracol vinculado a la reproducción asexual. Los rayos indican los puntos accesibles para la introducción de transgenes al parásito (mediante electroporación, microinyección, etc). Las flechas negras indican los procesos que ocurren naturalmente, y las blancas representan los procesos que resultan de la manipulación por el técnico. La línea de puntos indica los eventos ocurridos en el interior del caracol.*

3.2. Herramientas de manipulación genética en Schistosoma

Pionero en la manipulación génica de *Schistosomas* es el grupo de Davis y colaboradores quienes en 1999 bombardearon gusanos adultos de *S. mansoni* con ARNm y plásmidos conteniendo el gen codificante de luciferasa de luciérnaga (Davis *et al.* 1999). Posteriormente, Grevelding y colaboradores llevaron a cabo una serie de estudios empleando bombardeo de partículas en *S. mansoni*, utilizando plásmidos conteniendo genes reporteros fluorescentes bajo el control de promotores constitutivos como tejido específicos (por ejemplo, promotor de la catpesina F) de schistosomas (Wippersteg *et al.* 2002; Wippersteg *et al.* 2002; Rossi *et al.* 2003; Wippersteg *et al.* 2003; Wippersteg *et al.* 2005; Dvorak *et al.* 2010). Un plásmido conteniendo un gen codificante de la proteína verde fluorescente (GFP) bajo el control de los elementos reguladores del gen HSP 70 (del inglés "heat shock protein 70 kDa") de *Schistosoma* fue empleado para transfectar adultos y esporocistos de *S.mansoni* mediante bombardeo de partículas. No solo fue detectada la presencia del transgene mediante PCR en los tejidos de parásitos transfectados, sino que su expresión fue confirmada a nivel del ARNm por RT-PCR, y a nivel de proteína por Western blot y microscopía de fluorescencia (Wippersteg *et al.* 2002).

En el 2004 Correnti y Pearce, demuestran que es factible el empleo de la electroporación para transfectar ARNm de luciferasa de luciérnaga al interior de *Schistosomas* (Correnti and Pearce 2004). Esta técnica recibió gran aceptación y consecuentemente ha sido empleada para la transfección de plásmidos, ARN de doble cadena (ARNdc) y pequeños ARNs interferentes (siRNA- del inglés "short interfering RNAs") y otras moléculas reporteras tanto en *S.mansoni* [por ejemplo (Morales *et al.* 2008; Dvorak *et al.* 2010)] como en *S. japonicum* (Zhao *et al.* 2008). La electroporación ha sido empleada exitosamente para introducir ácidos nucleicos en esporocistos, schistosomulas y adultos de *Schistosomas*, frecuentemente empleando un pulso único de 125 V por 20 ms en cubetas de 4 mm [por ejemplo (Correnti *et al.* 2005; Faghiri and Skelly 2009)]. La electroporación fue también empleada para desarrollar un método relativamente sencillo y directo de detección de una vía funcional de ARNi mediante el

silenciamiento del gen reportero de la luciferasa de luciérnaga. Esta aproximación permite detectar una vía activa de ARNi en parásitos poco conocidos, donde la ARNi no ha sido descrita, como se demostró en *Fasciola hepatica*. Asimismo permite estudiar la actividad de moléculas interferentes menos convencionales como vectores codificantes de ARNs horquillas o shRNAs (del inglés "short hairpin RNAs") (Rinaldi *et al.* 2008; Ayuk *et al.* 2011).

Vectores plasmídicos y retrovirales también han sido utilizados con éxito para transfectar diferentes estadios de desarrollo de *S. mansoni*, demostrándose por primera vez la capacidad de integración de dichos vectores en el genoma de dicho parásito (Morales *et al.* 2007; Kines *et al.* 2008). En la actual era post-genómica de la biología de *Schistosoma* y a pesar de los promisorios avances que se han producido en el área de la genómica funcional, se hace necesario el desarrollo y optimización de herramientas que permitan dilucidar la función génica. La f**igura 7** representa la línea del tiempo enfatizando los principales avances en la manipulación génica de schistosomas de los últimos 15 años.

3.3. Aproximaciones de pérdida y ganancia de función génica

3.3.1. Interferencia de ARN

Estrategias experimentales que involucran perdida de función génica, como ARNi dirigido contra diversos genes de helmintos, en diversos estadios de desarrollo han sido reportadas (Skelly *et al.* 2003; Correnti *et al.* 2005; Dinguirard and Yoshino 2006; Osman *et al.* 2006). ARNi ha sido desarrollado y optimizado en diversos estadios y genes blanco en schistosomas, donde prácticamente su uso como herramienta de pérdida de función génica es rutinaria (revisado en (Geldhof *et al.* 2007; Kalinna and Brindley 2007; Krautz-Peterson *et al.* 2009). El primer reporte en *S.mansoni* mostró el silenciamiento de una catepsina B en schistosomulas mediante la simple incubación de los parásitos en presencia de la molécula interferente o "soaking" (Skelly *et al.* 2003).

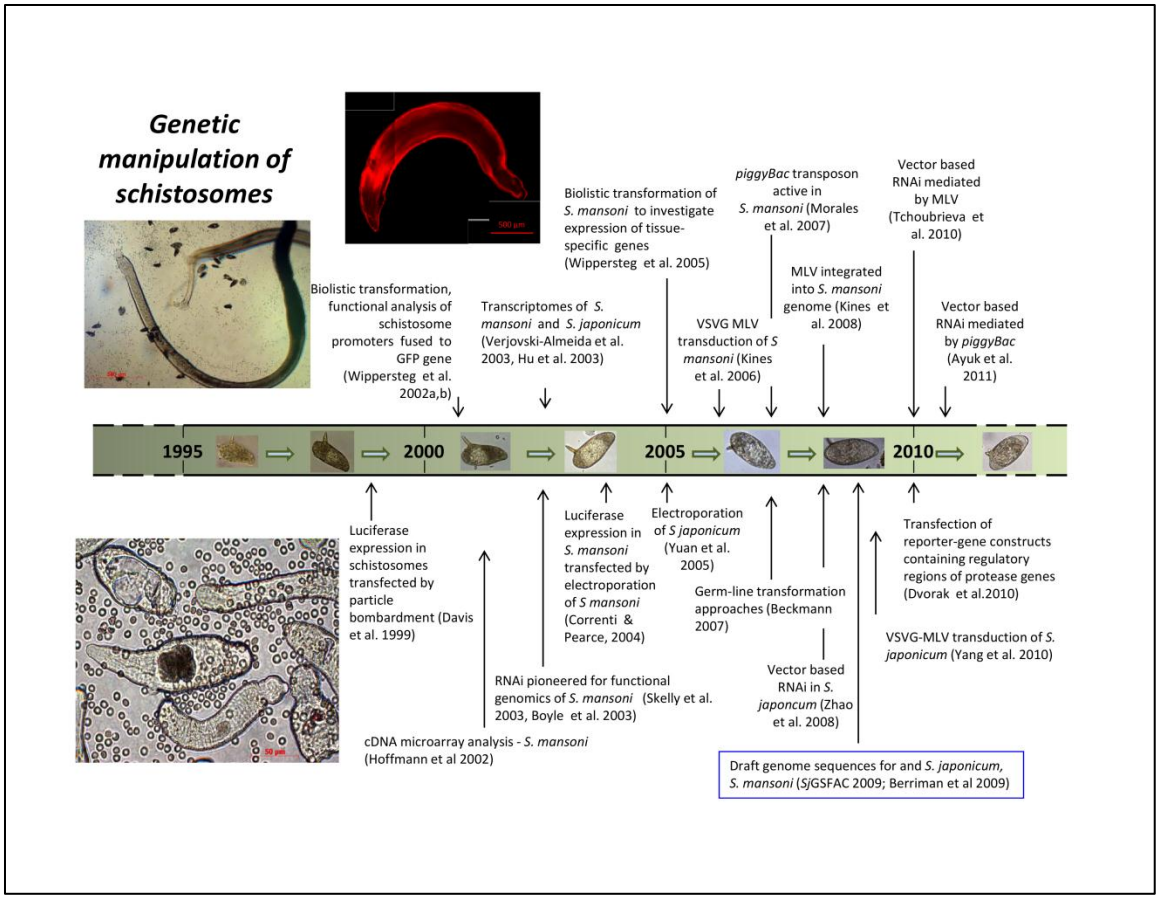

Figura 7. Línea del tiempo señalando los avances en la manipulación génica de los schistosomas. *Eventos pioneros reportados en los últimos 15 años son citados.*

Un estudio similar utilizando la electroporación como mecanismo de incorporación del ARNdc, mostró que el silenciamiento de catepsina B afecta el crecimiento de las schistosomulas, observándose que el efecto es persistente y progresivo (Correnti *et al.* 2005). La interferencia también fue ensayada en esporocistos (Boyle *et al.* 2003). Estos reportes iniciales demostrando la efectividad del ARNi en esta especie abrieron el camino al uso de la metodología como herramienta en el análisis de la función génica (Cheng *et al.* 2005; Delcroix *et al.* 2006; Dinguirard and Yoshino 2006; Osman *et al.* 2006; Krautz-Peterson *et al.* 2007; Morales *et al.* 2008).

Aunque las posibilidades de esta técnica aún distan bastante del uso sistemático del ARNi a escala genómica algunos avances se han obtenido en esta dirección. En este

sentido, Yoshino y colaboradores recientemente han reportado el silenciamiento de 32 genes diferentes en esporocistos cultivados *in vitro*, incluyendo antioxidantes, factores de transcripción, moléculas de señalización intracelular y enzimas involucradas en el metabolismo celular. Los resultados obtenidos indicaron que el nivel de silenciamiento génico producto del ARNi es muy variable y depende de: (1) el gen seleccionado a silenciar, (2) cuan accesible al ARNdc es el tejido donde se expresa el gen que se quiere silenciar, (3) la molécula de ARNdc empleada para inducir el silenciamiento génico (tamaño del ARNdc, sitio del ARNm que ha sido seleccionado para diseñar el ARNdc, etc) y (4) tiempo de evaluación de la interferencia luego del tratamiento con ARNdc (Mourao *et al.* 2009). El potencial efecto inespecífico del ARNi, como la variabilidad en la eficiencia del silenciamiento génico son puntos críticos que han de ser analizados y en lo posible controlados para cada estadio parasitario empleado, el tipo de molécula de ARNdc utilizada para inducir el silenciamiento, el gen que se quiere interferir, el mecanismo de transfección empleado (soaking, electroporación, etc), el tiempo de cultivo del parásito antes de la transfección con el ARNdc , y el tiempo transcurrido entre el tratamiento y el momento de evaluación del efecto interferente.

3.3.2. Transgénesis

La transgénesis implica la integración de una molécula de ácido nucleico exógeno al genoma del organismo en cuestión. Vectores con capacidad de integración al genoma como los transposones, han sido empleados para facilitar la manipulación de genomas de variados organismos (microorganismos, protozoarios, plantas y mamíferos). Ha sido establecida exitosamente la transgénesis estable de la línea germinal en genomas de invertebrados como *Plasmodium falciparum* (Balu *et al.* 2005), el mosquito vector de la fiebre amarilla *Aedes aegypti* (Jasinskiene *et al.* 1998), y el platelminto de vida libre planaria *Girardia tigrina* (Gonzalez-Estevez *et al.* 2003). En *S. mansoni* el transposon *piggyBac*, conteniendo el gen de la luciferasa de luciérnaga bajo el control del promotor de la actina de *Schistosoma*, fue empleado para transfectar schistosomulas. No solo se demostró a nivel de la secuencia génica que efectivamente el transposon se integró en el genoma de los parásitos transfectados, sino también que el gen reportero expresaba

activamente la enzima luciferasa de luciérnaga (Morales *et al.* 2007). Este fue el primer reporte que demostró por primera vez evidencia directa, a nivel de la secuencia de ADN, de la integración de un transgen en el genoma de *S. mansoni*. En el año 2006, el mismo grupo liderado por el Dr. Paul Brindley reportó el empleo de un vector retroviral, en particular el retrovirus de la leucemia murina modificado (MLV- del inglés "murine leukemia retrovirus") para transfectar diferentes estadios de desarrollo de *S. mansoni* (Kines *et al.* 2006). Dos años más tarde, el mismo grupo demostró que los transgenes retrovirales se integraron efectivamente en el genoma de los parásitos transfectados con MLV (Kines *et al.* 2008)

Retrovirus

Los retrovirus, virus de ARN, incluyen una gran familia de virus que infectan primariamente vertebrados, aunque también se los ha encontrado en otros organismos como moluscos o insectos (Fields *et al.* 1996; Leblanc *et al.* 2000; Syomin *et al.* 2001). Es probable que hayan evolucionado a partir de elementos endógenos móviles como los retrotransposones del tipo LTR (del inglés "long terminal repeat"-LTR-) (Malik *et al.* 2000; Malik and Eickbush 2001), adquiriendo la capacidad de generar partículas encapsuladas infecciosa. El genoma retroviral consiste en dos copias de ARN simple hebra con un tamaño aproximado de 10 kb, conteniendo genes fundamentales para completar su ciclo de vida (*gag*, *pol* y *env*), que codifican proteínas estructurales (cápside) y enzimáticas (retro-transcripción). La nucleocápside interna o "core" está compuesta por el producto de *gag* y transporta no solo el genoma viral sino también el producto enzimático de *pol* (la retrotranscriptasa viral o RT). La membrana viral contiene un único producto proteico codificado por *env*, y envuelve al "core". Esta proteína de cubierta determina la especificidad viral con respecto a la célula que infecta. La mayoría de los retrovirus interactúan con la célula huésped mediante un receptor de superficie que media la fusión de la membrana de cubierta viral con la membrana plasmática de la célula. Una vez en el interior del citoplasma el contenido de la nucleocápside viral (ARN genómico y RT) es liberado, y la enzima RT es utilizada para generar ADN copia a partir del genoma de ARN del virus. Es una ADN polimerasa-ARN dirigida, que utiliza como molde al ARN viral generando una copia de ADN o provirus que se transloca al núcleo

celular. Una vez en el interior del núcleo la enzima viral integrasa cataliza la inserción del provirus de ADN en el genoma de la célula huésped. Dicho provirus integrado en el genoma actúa de molde para generar los transcriptos de ARN que formarán el genoma de los nuevos virus **(Figura 8**). En el proceso de generación de nuevas partículas virales, algunas de ellas pueden transportar fragmentos de secuencias génicas derivadas del genoma de la célula huésped. La capacidad de los retrovirus para transportar en su genoma secuencias codificantes de la célula huésped hace que sean candidatos naturales como vectores para el desarrollo de transgénesis.

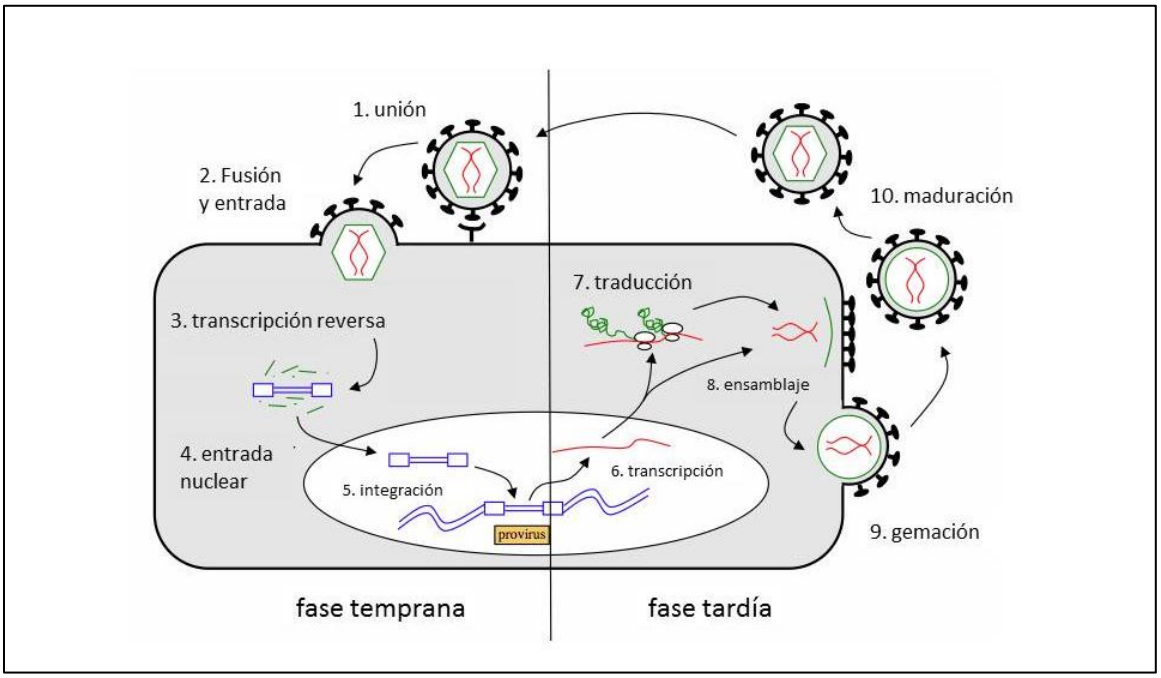

Figura 8. Ciclo de vida del retrovirus. *El ciclo de vida se divide arbitrariamente en dos fases, temprana y tardía. En la fase temprana del ciclo la partícula viral interactúa con la membrana plasmática de la célula huésped, libera su genoma de ARN en el citoplasma, que se retrotranscribe mediante la acción de la transcriptasa reversa viral para generar el ADN copia que se transloca al núcleo y finalmente se integra al genoma mediante la acción de la integrasa. Una vez integrado el provirus comienza a transcribirse (fase tardía) generando el genoma viral, molde de traducción de las proteínas virales que se ensamblan formando las partículas virales que finalmente emergen de la célula mediante la gemación a partir de la membrana plasmática. Adaptado de http://www.nimr.mrc.ac.uk/research/kate-bishop*

Virus de la Leucemia Murina modificado

El virus de la leucemia murina modificado (del inglés "pseudotyped moloney murine leukemia virus"-MLV) es un retrovirus murino, cuyo genoma codifica los productos *gag*,

pol y env. MLV ha sido empleado para facilitar la transferencia de genes en estudios de terapia génica (Anderson 1998). En los vectores MLV, algunas secuencias que codifican proteínas virales han sido reemplazadas por genes reporteros, mientras que los elementos reguladores implicados en la retrotranscripción y señales de empaquetamiento viral son mantenidos. Se han diseñado líneas celulares específicas (GP2-293, derivada de riñón humano) que expresan los genes estructurales virales, pero sin los elementos reguladores de los mismos, de tal forma que son incapaces de producir partículas virales infecciosas (Fields *et al.* 1996). La transfección de estas líneas celulares de empaquetamiento con plásmidos que codifican genes reporteros flanqueados por los elementos LTR del genoma viral, facilita la producción de partículas virales infecciosas pero incompetentes desde el punto de vista replicativo.

El rango de células huéspedes que es capaz de infectar MLV está determinado por la proteína de cubierta del virus y está básicamente restringido a células murinas lo que limita su empleo en células derivadas de organismos invertebrados. Para ampliar el rango de huéspedes susceptibles a la infección viral es posible intercambiar la proteína original de cubierta por otra proteína derivada de otro virus que tenga un rango mayor de hospederos, incluyendo organismos invertebrados. Este proceso, llamado "pseudotipado viral" y desarrollado en la década del 90, se ha optimizado y prácticamente en la actualidad es técnica de rutina en laboratorios que se dedican a transgénesis y terapia génica mediada por retrovirus (Emi *et al.* 1991). De esta forma se han diseñado vectores MLV modificados capaces de expresar la glicoproteína de cubierta del rabdovirus de la estomatitis vesicular (del inglés "vesicular stomatitis virus glycoprotein"-VSVG-) (Yee *et al.* 1994; Coll 1995; Ory *et al.* 1996). Esta proteína de cubierta viral (VSVG) es capaz de reconocer receptores de membrana más conservados y por lo tanto presentes en casi todos los organismos eucariotas. Además, como VSVG es expresado como un único péptido (la proteína de cubierta original de MLV presenta dos subunidades), el vector viral es sencillo de producir mediante la co-transfección de las líneas celulares productoras con el plásmido que transporta los genes reporteros-exógenos flanqueados por los elementos LTRs, junto con un plásmido que codifica VSVG. El virus MLV

modificado es relativamente sencillo de producir, concentrar y manipular sin pérdida de su capacidad infectiva (Burns *et al.* 1993).

Retrovirus modificados han sido utilizados para infectar, integrar secuencias exógenas y expresar genes reporteros en varios organismos vertebrados e invertebrados como peces, oocitos de *Xenopus*, salamandra, varios moluscos, protozoarios como ameba, insectos como mosquitos y *Drosophila* (Burns *et al.* 1994; Lin *et al.* 1994; Lu *et al.* 1996; Matsubara *et al.* 1996; Franco *et al.* 1998; Jordan *et al.* 1998; Que *et al.* 1999; Boulo *et al.* 2000).

Brindley y colaboradores han realizado un avance sustancial en la transgénesis de *S. mansoni* utilizando el retrovirus MLV modificado (Kines *et al.* 2006; Kines *et al.* 2008). Se investigó la posibilidad de utilizar MLV expresando la proteína VSVG de superficie, para infectar, transducir genes reporteros y estudiar su expresión en diferentes estadios de desarrollo del parásito (Kines *et al.* 2006). En particular, schistosomulas fueron expuestas al virus y el ADN genómico fue extraído al cabo de 5 días. Análisis por Southern blot indicaron la presencia del provirus en el genoma de los parásitos transducidos. Fragmentos de secuencia del transgen flanquedos por secuencia genómica de *S. mansoni* fueron identificados utilizando RAP (del inglés "retrotransposon anchored PCR"), técnica desarrollada en el laboratorio del Dr. Brindley. Se demostró a nivel de secuencia de ADN la integración de la secuencia del provirus en el genoma del parásito transducido con MLV. La expresión de los genes reporteros bajo la regulación del promotor de actina también fue investigada, mediante el análisis de actividad luciferasa en schistosomulas y adultos transducidos con viriones. La **figura 9** representa el sistema de producción de MLV modificado que es empleado para transfectar células de *Schistosoma*.

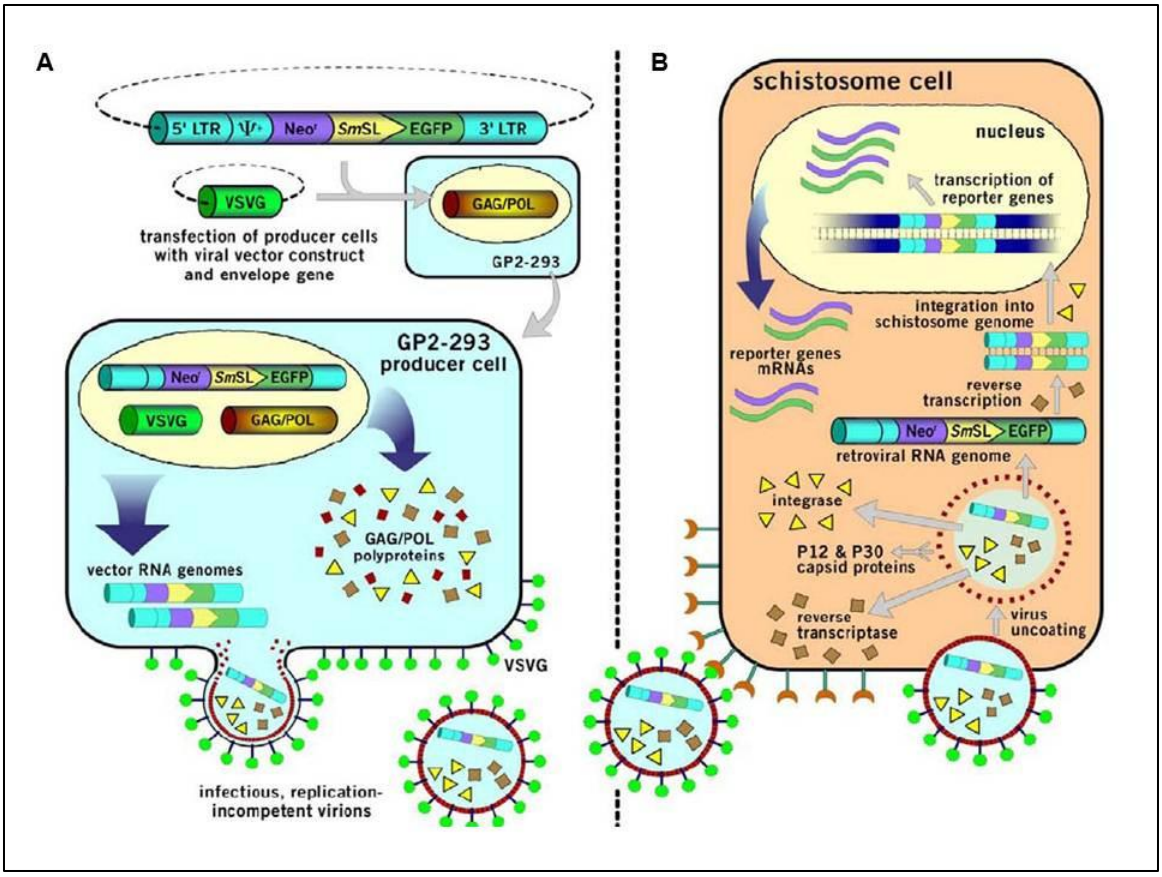

Figura 9: Representación esquemática de la producción del virus de la leucemia murina modificado (MLV) y transducción de schistosomas. *Panel A: El vector viral es modificado conteniendo un promotor de S. mansoni (Splice Leader) adyacente al gen de la proteína verde fluorescente como reportero (EGFP). Las células productoras GP2-293 que contienen en su genoma los genes Gag-Pol son cotransfectadas con el vector viral modificado y un plásmido conteniendo el gen de cubierta del rabdovirus de la estomatitis vesicular (del inglés "vesicular stomatitis virus glycoprotein"-VSVG-). Las partículas virales infectivas, no competentes desde el punto de vista reproductivo son producidas y colectadas desde el sobrenadante celular. (B) Los schistosomas son infectados con las partículas virales. El genoma de ARN del retrovirus es retrotranscripto a ADN que luego se integra al genoma del parásito como provirus. El ARNm transcripto a partir del provirus integrado se expresa, es exportado al citoplasma donde se traduce en la proteína reportera (de (Kines et al. 2006).*

La transgénesis de schistosomas mediada por vectores retrovirales ofrece la posibilidad de integrar secuencias génicas exógenas en el genoma del parásito en estudios de ganancia y/o pérdida de función. La expresión de genes reporteros y/o genes que confieran una propiedad nueva determinando algún fenotipo en particular [por ej. genes de resistencia a drogas (Giordano-Santini *et al.* 2010; Semple *et al.* 2010)] son solo algunos ejemplos de estudios de ganancia de función génica en *Schistosoma*s. Dado que

el genoma de *S. mansoni* se encuentra disponible, análisis a mayor escala como la mutagénesis insercional mediante vectores retrovirales son posibles (Langridge *et al.* 2009). La generación de retrovirus cuyo genoma contenga un cassette codificante de un ARN horquilla dirigido contra genes reporteros o genes endógenos relevantes, es un ejemplo del empleo de la transgénesis en estudios de perdida de función génica. La expresión de dichos cassettes puede estar controla por promotores constitutivos, tejido específicos o incluso condicionales (Ortiz and Johnson 2003; Nishijima *et al.* 2009; Dvorak *et al.* 2010).

3.3.3. Interferencia de ARN mediada por vectores

El silenciamiento génico por ARNi mediado por vectores codificantes de pequeñas horquillas de ARN (shRNAs) ha sido exitosamente ensayado en *Schistosoma*. En el 2008 Zhao y colaboradores reportan el silenciamiento génico del gen Mago nashi mediante siRNAs generados *in vivo* a partir de shRNAs codificados en un vector plasmídico bajo el promotor H1 de la ARNpol III en schistosomulas de *S. japonicum* (Zhao *et al.* 2008). Asimismo, Tchoubrieva y colaboradores lograron silenciar el gen de la catepsina B1 en *S. mansoni* mediante un vector retroviral que expresa shRNAs bajo el control del promotor de la actina, en adultos de *S. mansoni* (Tchoubrieva *et al.* 2010) (**Figura 10**). A pesar de la utilidad de la técnica de ARNi convencional, mediada por ARNdc, generalmente el efecto de silenciamiento es transitorio y muchas veces es ineficiente para algunos genes expresados en determinados estadios de desarrollo del parásito y/o tejido.

Las aproximaciones de ARNi mediada por shRNAs codificados en vectores plasmídicos y particularmente vectores retrovirales que pueden integrarse en el genoma pueden determinar un efecto continuo, más prolongado en el tiempo, trans-generacional, y en todas las células del organismo. Su expresión puede ser continua o controlada bajo un promotor condicional (ter Brake et al., 2006; Sliva and Schnierle, 2010). El desarrollo de la transgénesis somática, pero fundamentalmente de la línea germinal ofrece así una oportunidad única para desarrollar este tipo de tecnología de perdida de función continua y/o condicional de genes relevantes y potenciales blancos de nuevas drogas y/o vacunas.

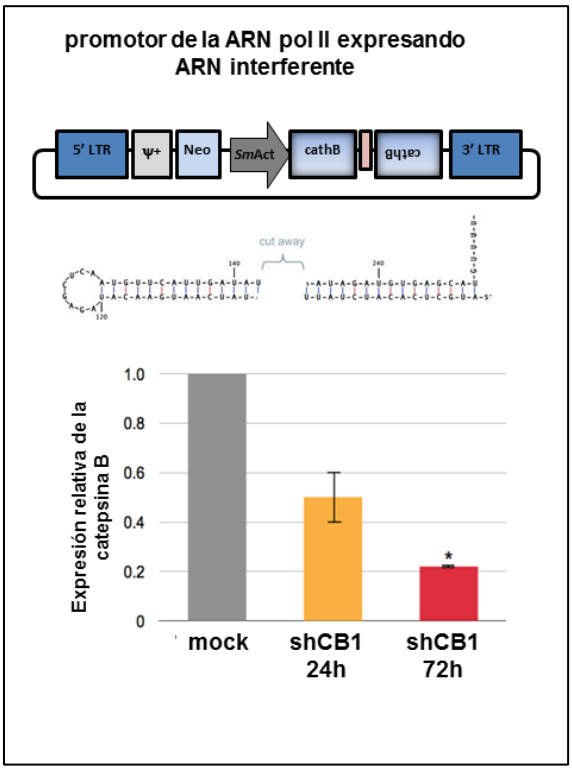

Figura 10. ARN de interferencia (ARNi) basado en vectores codificantes "shRNAs". *ARNi mediado por largos hairpin ARNs (hpRNAs) expresados bajo el control de promotores de la ARN polimerasa II (promotor de la actina de S. mansoni) en el vector retroviral del virus de la leucemia murina modificada (MLV) para silenciar la catepsina B1 de S. mansoni. Arriba: Representación esquemática del vector retroviral y el hpRNA. Abajo: Silenciamiento de la catepsina B en schistosomas transducidos con MLV (Tchoubrieva et al. 2010).*

Hallazgos reportados por el grupo de Grevelding son pioneros en cuanto a la introducción de transgenes en la línea germinal de *Schistosomas* (Grevelding 2006; Beckmann *et al.* 2007). Miracidios transfectados mediante bombardeo de partículas con plásmidos codificantes de GFP fueron empleados para infectar caracoles *Biomphalaria glabrata*. La presencia del transgen codificante de GPF fue detectada no solo en las cercarias emitidas por dichos caracoles (generación F0), sino también por los adultos y huevos obtenidos de hámsteres infectados con dichas cercarias (generación F1). En las generaciones F2 y F3 ya no fue posible detectar la presencia del transgen, probablemente debido a un efecto de dilución y/o degradación del plásmido a lo largo de las

generaciones. A pesar del efecto de dilución y pérdida del plásmido a partir de la generación F2, estos hallazgos demostraron que es posible transfectar células germinales del parásito presentes en el huevo y miracidio y que posteriormente darán origen en los estadios intracaracol a las cercarias, e indican por primera vez que la transgénesis de la línea germinal en *Schistosoma mansoni* es posible.

Estos antecedentes promisorios en *S. mansoni* sugieren que estas herramientas de genómica funcional deben desarrollarse y optimizarse no solo en este parásito, sino en otros trematodos menos conocidos desde el punto de vista molecular. Esto redundará en la generación de estrategias de control parasitario tan necesarias en estas parasitosis desatendidas, objetivo fundamental en la era post-genómica de la parasitología molecular.

HIPOTESIS Y OBJETIVOS

Hipótesis

Considerando las particularidades del ciclo de vida de los trematodos, es posible generar modificaciones genéticas estables y trans-generacionales en estos parásitos. Estas herramientas de estudio de función génica pueden desarrollarse y optimizarse en *Schistosoma mansoni*, por ser el parásito modelo más estudiado, y luego ser transferidas a otras especies menos conocidas.

Esto permitirá no solo responder preguntas sobre la biología básica de dichos parásitos, sino que también facilitará el desarrollo *a posteriori* de nuevas estrategias de intervención y control parasitario.

Objetivo general

Nos planteamos como **objetivo general** del siguiente trabajo la optimización de la transgénesis estable y trans-generacional mediada por vectores retrovirales y la interferencia de ARN convencional (mediada por ARN doble cadena) y no convencional (mediada por vectores codificantes de horquillas de ARN) en diversos estadios de desarrollo del parásito trematodo *Schistosoma mansoni*. Una vez desarrolladas y optimizadas, dichas herramientas de genómica funcional serán transferidas a otros parásitos modelos menos estudiados como *Schistosoma haematobium y Fasciola hepatica*.

Objetivos específicos

Para cumplir con dicho objetivo nos planteamos los siguientes **objetivos específicos**.

A. Optimización de la transgénesis en Schistosoma mansoni

A.1 Cuantificación de transgenes en parásitos transducidos con vectores retrovirales

A.2 Evaluación de la integración genómica de los transgenes

A.3 Selección de organismos transgénicos

A.4 Análisis de la expresión de genes reporteros en parásitos transgénicos

B. Evaluación de la transfección estable de células germinales

B.1 Estudio de la viabilidad para generar modificaciones genéticas en huevos de *Schistosoma mansoni*

B.2 Evaluación de la transfección y transgénesis en huevos de *S. mansoni*

B.3 Desarrollo de herramientas de pérdida de función génica estable mediante la transgénesis con vectores codificantes de ARNs horquillas

C. Transferencia de tecnología a otros parásitos trematodos

C.1 Generación de herramientas de estudio de función génica en *Schistosoma haematobium*

C.2 Desarrollo de la transgénesis mediada por vectores retrovirales en *Fasciola hepatica*

ESTRATEGIA GENERAL

A. Optimización de la transgénesis en *Schistosoma mansoni*

Si bien se viene investigando desde hace tiempo en la transgénesis en *Schistosoma,* y se han logrado avances muy significativos, quedan aún varios aspectos sin resolver que son relevantes para establecer la técnica como rutina. A diferencia de los ensayos con líneas celulares de mamíferos, se trabaja con parásitos enteros, por lo que en un experimento varia no solamente la proporción de parásitos efectivamente tratados, sino también los niveles de incorporación, y la localización y/o células o tejidos que fueron efectivamente transfectados. Es relevante consecuentemente encontrar métodos que permitan seleccionar la población transfectada, cuantificar la incorporación del transgene, y localizar el mismo tanto a nivel celular como a nivel genómico. En este trabajo abordamos algunos de estos aspectos.

A.1. Cuantificación de transgenes en parásitos transducidos con vectores retrovirales

El virus de la leucemia murina modificado (MLV) es capaz de transducir diferentes estadios de *S. mansoni* e integrarse en su genoma (Kines *et al.* 2006; Kines *et al.* 2008). Se han identificado las secuencias del provirus en el genoma de los parásitos transducidos con MLV mediante RAP (del inglés "Retrotransposon anchored PCR"), una técnica basada en PCR que incluye juegos de cebadores que reconocen secuencias repetidas endógenas del genoma parasitario y cebadores específicos del transgen (Morales *et al.* 2007; Kines *et al.* 2008). Si bien el RAP permitió clonar y secuenciar el provirus de MLV integrado al genoma de *S. mansoni*, es una técnica cualitativa que no permite determinar la cantidad de viriones que efectivamente transfectaron a los parásitos, ni el número de copias de transgenes integrados en el ADN. ¿Es posible emplear PCR en tiempo real para estimar el número de copias del transgen en el ADN genómico de parásitos transducidos con el retrovirus MLV? Basados en (1) el RAP y (2) en el Alu-PCR, técnica desarrollada para cuantificar el número de copias del provirus de HIV integrado en los cromosomas de células humanas, desarrollamos una aproximación cuantitativa por PCR en tiempo real

para estimar el número de copias del transgen por nanogramo de ADN genómico aislado de parásitos transducidos con MLV.

A.2. Evaluación de la integración genómica de los transgenes

Las estrategias de secuenciado a gran escala utilizando secuenciadores de la nueva generación han permitido realizar estudios de mutagénesis insercional en diversos organismos, no solo detectando los sitios preferenciales de inserción del vector integrable sino también asignando roles biológicos a una enrome cantidad de secuencias génicas (Langridge *et al.* 2009). En particular se ha demostrado que en células de mamíferos MLV tiene la tendencia a integrarse en las regiones 5' de los genes, mientras que HIV lo hace en las unidades transcripcionales a lo largo de toda la secuencia codificante (Bushman *et al.* 2005; Lewinski *et al.* 2006). Nos preguntamos si es posible emplear aproximaciones de secuenciado a gran escala (por ej. Illumina) para identificar y definir los sitios preferenciales de integración de MLV en el genoma de schistosomas transducidos con los viriones. ¿MLV se integra preferentemente en ciertas regiones del genoma de *Schistosoma*, o por el contrario la integración es aleatoria? ¿Difieren los sitios de integración de MLV en un modelo de organismo invertebrado, como lo son los *Schistosomas*, en comparación con lo que sucede en células de mamífero? ¿Cómo es la distribución a escala genómica de los sitios de inserción de MLV con respecto a las secuencias codificantes, no codificantes, repetidos, islas CpG, regiones reguladoras, etc? Para contestar estas preguntas decidimos emplear secuenciado a gran escala mediante Illumina de ADNs genómicos aislados de parásitos transducidos con MLV. El ADN se fragmenta, se ligan adaptadores y se procede a la generación de bibliotecas de Illumina mediante el empleo de cebadores dirigidos contra los adaptadores y contra la secuencia del provirus. Las librerías se secuencian y analizan utilizando la secuencia del genoma completo de *Schistosoma mansoni* como referencia (Berriman *et al.* 2009), para identificar los sitios de integración del provirus en el genoma de los parásitos transducidos.

A.3. Selección de organismos transgénicos

La selección mediante el uso de marcadores de resistencia a drogas (como por ej. antibióticos) de células transfectadas/transgénicas es de uso rutinario cuando se trabaja con bacterias y/o líneas celulares. *Caenorhabditis elegans* es el modelo helminto para el cual las herramientas de manipulación génica como la transgénesis están bien avanzadas. Sin embargo, recién en el año 2010 se reportaron dos trabajos que muestran que es factible la selección de líneas de gusanos transgénicos mediante el uso de genes marcadores de resistencia a antibiótico (Giordano-Santini *et al.* 2010; Semple *et al.* 2010). Nos preguntamos si es posible transducir schistosomas con MLV, cuyo genoma codifica el gen de resistencia a la neomicina (*neoR*), y posteriormente seleccionar la población de organismos transfectados/transgénicos mediante el uso del antibiótico aminoglucósido geneticina (G418) en el medio de cultivo. Para ello, en primer lugar evaluamos diferentes concentraciones de la droga y tiempos de cultivo para generar curvas de sobrevida parasitaria *in vitro.* Posteriormente comparamos la sobrevida de parásitos transducidos con MLV con la sobrevida de parásitos controles no tratados, ambos expuestos en cultivo a la geneticina. A nivel molecular estudiamos la presencia y la expresión del transgen *neoR* en los parásitos transducidos por MLV.

A.4. Análisis de la expresión de genes reporteros en parásitos transgénicos

Reportes previos de Brindley y colaboradores sugieren que genes reporteros integrados en el genoma de schistosomas se expresan activamente en parásitos transducidos con MLV (Kines *et al.* 2006; Kines *et al.* 2008) o transfectados con el transposon *piggyBac* (Morales *et al.* 2007). ¿Cuán activo son los genes reporteros expresados en parásitos transducidos con MLV? ¿Cómo es el perfil temporal de expresión de los genes reporteros? Dado que en sistemas retrovirales empleados en terapia génica se ha reportado una expresión variable e incluso silenciamiento de los transgenes integrados en el genoma (Malik and Arumugam 2005), nos preguntamos si en schistosomas transducidos con MLV se puede observar un fenómeno similar. De existir silenciamiento en la expresión de los transgenes retrovirales, ¿es posible que cambios en

la estructura de la cromatina como modificaciones de histonas o metilación del ADN, puedan estar involucrados? Recientemente se ha reportado que el genoma de *Schistosoma mansoni* presenta metilación (Geyer *et al.* 2011), lo que sugiere la posibilidad de eventos epigenéticos de regulación de la expresión génica en estos parásitos. De ser así, ¿es posible rescatar y conservar en el tiempo la expresión de transgenes mediante el uso de secuencias aislantes (insulators) integradas al vector retroviral? Para responder algunas de estas preguntas clonamos secuencias aislantes de cromatina en los vectores retrovirales y luego de transfectar parásitos con dichos vectores analizamos los niveles de expresión de genes reporteros para evaluar el efecto de silenciamiento o rescate de la expresión génica debido a la presencia de secuencias aislantes de la cromatina (Chung *et al.* 1993; Arumugam *et al.* 2009).

B. Evaluación de la transfección estable de células germinales

La transgénesis en *Schistosomas* se ha ensayado mayoritariamente en el estadio infectivo del hombre, la schistosomula. Si bien es posible determinar la incorporación del transgen al genoma, la posibilidad de establecer líneas transgenicas estables depende de acceder a la línea germinal. Las particularidades del complejo ciclo vital de los trematodos ofrecen una ventana interesante donde lograr este objetivo. Normalmente el desarrollo en los estadios intra-caracol se hace de forma asexual por amplificación presumiblemente clonal a partir de masas de células indiferenciadas, por lo que de lograr transfectar estas células, se podría lograr una transfección estable trans-generacional. Para avanzar en esa dirección es necesario dirigir los esfuerzos en los estadios previos es decir, huevos y miracidios, por lo que en este estudio comenzamos a evaluar la viabilidad de intervenir en estos estadios.

B.1. Viabilidad de generar modificaciones genéticas en huevos de Schistosoma mansoni

Los huevos de *S. mansoni* son ricos en células germinales a partir de las cuales y a través de una expansión clonal de los estadios intracaracol se van a generar cercarias (Jurberg *et al.* 2009). Por este motivo este estadio del desarrollo representa una excelente ventana para incorporar transgenes al ciclo de vida del parásito y propagarlo de manera estable y transgeneracional. En primer lugar, nos preguntamos si es viable la manipulación génica de huevos mediante la incorporación de ARN doble cadena. Para ello llevamos a cabo experimentos de interferencia de ARN (ARNi) para estudiar específicamente el rol funcional de proteasas parasitarias en huevos. La actividad de enzimas leucinaminopeptidasas (LAPs) fue detectada en huevos de *Schistosoma manosni* hace más de 30 años (Bogitsh and Carter 1975; Bogitsh 1983) y estudios histoquímicos más recientes localizaron LAPs en huevos y en el infiltrado inflamatorio producido por los huevos en el tejido hepático de ratones infectados experimentalmente (Abouel-Nour *et al.* 2005). Por otro lado existe evidencia experimental que la actividad de la LAP está vinculada al proceso de eclosión de los huevos del parásito, ya que el tratamiento de huevos con bestatina (inhibidor de las LAPs) redujo significativamente la eclosión y la emergencia de los miracidios (Xu and Dresden 1986). Consecuentemente nos preguntamos si es posible emplear ARNi para comprender la función de dichas enzimas en los huevos. Generamos ARNs doble cadena largos dirigidos contra dos LAPs que habíamos identificado, aislado y clonado. Mantuvimos huevos de *S. mansoni* en cultivo en presencia de los ARNs doble cadena, y evaluamos el efecto interferente a nivel de ARNm, proteína y fenotípico.

B.2. Evaluación de la transfección y transgénesis en huevos de S. mansoni

Dadas las posibilidades que ofrece trabajar con los huevos de *S. mansoni*, ¿es posible introducir transgenes en las células germinales mediante la transducción de huevos del parásito con viriones de la leucemia murina modificada (MLV)? Una vez integrado el

transgen en el genoma de las células germinales, ¿es posible la transmisión del mismo a través de diversos estadios de desarrollo a lo largo de distintas generaciones?

Para comenzar a responder estas preguntas diseñamos una serie de experimentos de transfección de huevos con una serie de moléculas reporteras incluyendo pequeños ARNs doble cadena conjugados a un fluorocromo y ARNm de la luciferasa de luciérnaga sintetizado *in vitro*. Finalmente transfectamos huevos con MLV y comparamos la eficiencia de transducción entre la electroporación de huevos con viriones y el simple cultivo de los huevos en presencia de los viriones.

B.3. Desarrollo de herramientas de perdida de función génica estable mediante la transgénesis con vectores codificantes de ARNs horquillas

Recientes reportes demuestran que es posible inducir silenciamiento génico en schistosomas mediante el empleo ARNs horquilla (del inglés "short hairpin RNAs" o shRNAs) codificados en vectores plasmídicos o retrovirales (Zhao *et al.* 2008; Tchoubrieva *et al.* 2010). Estos hallazgos abren la posibilidad de generar líneas de parásitos transgénicos que expresen shRNAs de manera estable y transgeneracional. Zhao y colaboradores emplean shRNAs codificados en plásmidos bajo el control del promotor de la ARN pol III, H1. A pesar que logran un efecto de silenciamiento significativo con un evidente efecto fenotípico, los shRNAs se generan a partir del plásmido en forma episómica cuyo efecto es eventualmente transitorio (Zhao *et al.* 2008). Tchoubrieva y colaboradores emplearon el virus de la leucemia murina modificado (MLV) codificante de un ARN horquilla dirigido contra la enzima catepsina B1 de *S. mansoni* bajo el control del promotor de la actina (un promotor de ARN pol II) (Tchoubrieva *et al.* 2010). Con el objetivo de establecer una herramienta de silenciamiento génico que permita realizar estudios de perdida de función mediada por shRNAs en forma estable y transgeneracional, nos preguntamos si es factible el empleo de vectores integrables en el genoma (como MLV y transposon *piggyBac*) codificantes de ARNs horquilla bajo el control de promotores de la ARN pol III para inducir silenciamiento en *S. mansoni*. ¿Es posible inducir silenciamiento con ARNs horquilla expresados bajo el control del promotor de la ARN pol III U6 de schistosoma? ¿Cuán eficiente es la secuencia

promotora de U6 humana en comparación con la de schistosoma para inducir silenciamiento génico medido por ARNs horquilla en *S. mansoni*?

Para esto, identificamos y clonamos las secuencias promotoras del gen U6 de *Schistosoma mansoni* y humana en dos vectores integrables, piggyBac y pLNHX (el vector utilizado para generar las partículas virales MLV), junto a un cassette codificante de ARN horquilla dirigido contra el gen reportero de la luciferasa de luciérnaga. Posteriormente llevamos a cabo una serie de experimentos de transfección de los vectores productores de ARNs horquillas (piggyBac y MLV) en schistosomas, evaluemos el efecto interferente de los mismos y comparamos el silenciamiento génico producido por las horquillas de ARN expresadas a partir del promotor de schistosoma y el promotor humano.

Estos estudios son el resultado de una colaboración con otros estudiantes de postgrado del laboratorio y constituyen parte de su trabajo de tesis, por lo que se incluyen aquí como anexo 1.

C. Transferencia de tecnología a otros parásitos trematodos

Las herramientas de genómica funcional en desarrollo en *Schistosoma mansoni* pueden ser útiles para el estudio de otros trematodos. Desde esta perspectiva un blanco interesante es la tercera especie de *Schistosoma*, *S.haematobium*, y también especies responsables de trematodiosis digestivas. Allí se destacan las trematodiosis biliares causadas por *Opistorchis y Clonorchis*, de gran relevancia en el sudeste asiático, y la fascioliasis, zoonosis de distribución mundial con gran impacto ganadero en nuestro país.

C.1. Generación de herramientas de estudio de función génica en Schistosoma haematobium

El impacto en salud pública producido por *Schistosoma haematobium*, agente responsable de la schistosomiasis urinaria, supera al producido por *S. mansoni* y *S. japonicum* combinados. A esto se le suma la asociación entre la infección por *S.*

haematobium con (1) el carcinoma escamoso de vejiga y (2) el aumento en la susceptibilidad de la infección por HIV. Sin embargo *S. haematobium* es el schistosoma menos estudiado desde el punto de vista molecular, no se cuenta aún con el genoma completo y no se han desarrollado las herramientas de estudio de función génica (Rollinson 2009). Dado el desarrollo de tecnología de genómica funcional aplicada a *S. mansoni*, ¿es posible transferir dicha tecnología a *S. haematobium*, cultivar diversos estadios del parasito, realizar transección de genes reporteros y realizar manipulación génica? Finalmente, ¿existe una vía de ARNi activa en este parasito? Para responder estas preguntas, en primer lugar obtuvimos, cultivamos y transfectamos con moléculas reporteras diversos estadios de desarrollo de *S. haematobium*. Empleando ARN doble cadena intentamos silenciar el ARNm reportero de la luciferasa de luciérnaga siguiendo un protocolo previamente desarrollado por nuestro grupo de trabajo (Rinaldi *et al.* 2008). Finalmente realizamos experimentos de silenciamiento génico mediante ARNi contra un gen endógeno de *S. haematobium*.

C.2. Desarrollo de la transgénesis mediada por vectores retrovirales en Fasciola hepatica

En el año 2008 transferimos exitosamente conocimiento y tecnología de genómica funcional de *Schistosoma mansoni* a *Fasciola hepatica*. Luego de probar diferentes condiciones de cultivo, logramos mantener por más de un mes juveniles de *F. hepatica* desenquistados *in vitro* a partir de metacercarias, en medio empleado para mantener schistosomulas de *S. mansoni* en presencia o en ausencia de glóbulos rojos lavados (Rinaldi 2008). Transfectamos juveniles recientemente desenquistados de *F. hepatica* (JRD) con diversas moléculas reporteras mediante electroporacion. Asimismo, empleando schistosomulas como un control positivo diseñamos un protocolo para la evaluación de la presencia de una vía activa de ARNi en JRD mediante el silenciamiento por ARN doble cadena del ARNm reportero de la luciferasa de luciérnaga. Finalmente pudimos silenciar por ARN doble cadena a la leucinaminopeptidasa de *F. hepatica* como ejemplo de un gen endógeno (Rinaldi *et al.* 2008). Sabiendo que la transgénesis es factible en schistosomas y basados en nuestra experiencia previa de transferencia de

tecnología de una especie a otra, nos planteamos desarrollar la transgénesis mediada por el virus de la leucemia murina modificada (MLV) en juveniles de *F. hepatica*. Considerando las similitudes, pero también las diferencias de los ciclos de vida y características biológicas entre las especies de trematodes del genero *Schistosoma* y *Fasciola*, nos preguntamos si es posible transducir con MLV a los JRD de *F. hepatica*, y detectar la presencia y la expresión de los transgenes en los parásitos tratados. Para ello llevamos a cabo experimentos de desenquiste, cultivo e infección de JRD con viriones MLV. Estudiamos mediante inmunohistoquímica la fijación de las partículas virales a la superficie de los juveniles tratados y analizamos posteriormente la presencia del transgen en el ADN genómico.

MATERIALES Y METODOS

Dado que la mayoría de los resultados presentados en el presente trabajo ya se han publicado en revistas arbitradas internacionales o han sido enviados para que se considere su publicación, los materiales y métodos están descriptos en las secciones correspondientes de los artículos presentados. En el presente capítulo solo describiremos aquellos materiales y métodos correspondientes a resultados aún no publicados.

A. Evaluación de la integración genómica de los transgenes en *Schistosomas* transducidos con virus de la leucemia murina modificado (MLV)

Estadios de desarrollo de Schistosoma mansoni

Ratones y caracoles de la especie *Biomphalaria glabrata* infectados con la cepa de *Schistosoma mansoni* de Puerto Rico fueron suministrados por el Dr. Fred Lewis, Biomedical Research Institute, Rockville, MD. Los adultos de *S. mansoni* fueron obtenidos por perfusión de la vena porta de los ratones infectados siguiendo un protocolo aprobado por el "Institutional Animal Case and Use Committee of the George Washington University" (Mann *et al.* 2010). Se obtuvieron huevos del parásito a partir de dos fuentes diferentes. Por un lado los huevos se aislaron de los hígados de los ratones infectados siguiendo el protocolo descrito en (Dalton *et al.* 1997). Por otro lado se colectaron y concentraron los huevos emitidos por gusanos adultos mantenidos en cultivo durante las primeras 48hs. luego de la perfusión de los ratones siguiendo el protocolo descripto en (Mann *et al.* 2011). Estos huevos, que denominamos "IVLE" (del inglés "*In Vitro* Laid Eggs"), en el momento de su obtención se encuentran en un estadio mucho más inmaduro de desarrollo, donde ocurren las primeras divisiones mitóticas del cigoto (Jurberg *et al.* 2009), en comparación con los huevos aislados de los hígados (Mann *et al.* 2011). Schistosomulas de *S. mansoni* se obtuvieron mediante transformación mecánica de cercarias emitidas por los caracoles infectados y se mantuvieron en cultivo según los protocolos desarrollados en (Mann *et al.* 2010).

Virus de la leucemia murina modificado, vectores retrovirales y transducción de schistosomas

El virus de la leucemia murina modificado expresando en su superficie la glicoproteína del virus de la estomatitis vesicular (del inglés "Vesicular stomatitis virus glycoprotein" o VSVG) fue producido utilizando los vectores pLNHX parental, pLNHX_SmAct-Luc, pLNHX_SmAct-GFP y pLNHX_SLGFP descriptos en (Kines *et al.* 2006), concentrado y cuantificado siguiendo el protocolo descripto en (Mann *et al.* 2011).

Schistosomulas y adultos de *S. mansoni* fueron transducidos con viriones MLV siguiendo los protocolos descritos en (Kines *et al.* 2006; Kines *et al.* 2008) y procesados para extracción de ADN genómico 48 hs post tratamiento. Los huevos obtenidos a partir de hígados de ratones infectados o emitidos *in vitro* por gusanos en cultivo fueron infectados con MLV según se describe en el artículo (Kines *et al.* 2010) presentado en el capítulo de Resultados y Discusión. Los huevos expuestos a MLV se cultivan hasta madurar, eclosionan y los miracidios resultantes son utilizados para infectar caracoles. Al cabo de 40 días aproximadamente, las cercarias emitidas por dichos caracoles son recolectadas y procesadas para extracción del ADN.

Bibliotecas de secuenciación Illumina

Para la preparación de las bibliotecas de Illumina se siguieron los protocolos desarrollados en (Quail *et al.* 2009). Brevemente, el ADN genómico aislado de schistosomulas, adultos y cercarias fue fragmentado mecánicamente por sonicación, obteniendo fragmentos de ~200 pb. Los fragmentos fueron preparados para la ligación de adaptadores mediante la reparación de los extremos y el agregado de Adeninas siguiendo las instrucciones del kit NEBNext™ DNA Sample Prep Reagent Set 1 de New England Biolabs (E6000B, NEB Ipswich, MA, USA). Los fragmentos de ADN ligados a los adaptadores se amplificaron por nested-PCR utilizando cebadores específicos de la región LTR del extremo 5' o 3' del provirus (del inglés "long terminal repeat" o LTR) y cebadores que reconocen la secuencia del adaptador, generando una biblioteca para cada extremo del transgen (biblioteca 5' y 3'). Los productos de amplificación fueron

separados y purificados en un gel de agarosa, cuantificados por PCR en tiempo real y sembrados en la placa de Illumina para su amplificación y posterior secuenciación. La **figura 11** resume la estrategia de preparación y secuenciado de las bibliotecas de Illumina preparadas con el ADN proveniente de parásitos transducidos con MLV.

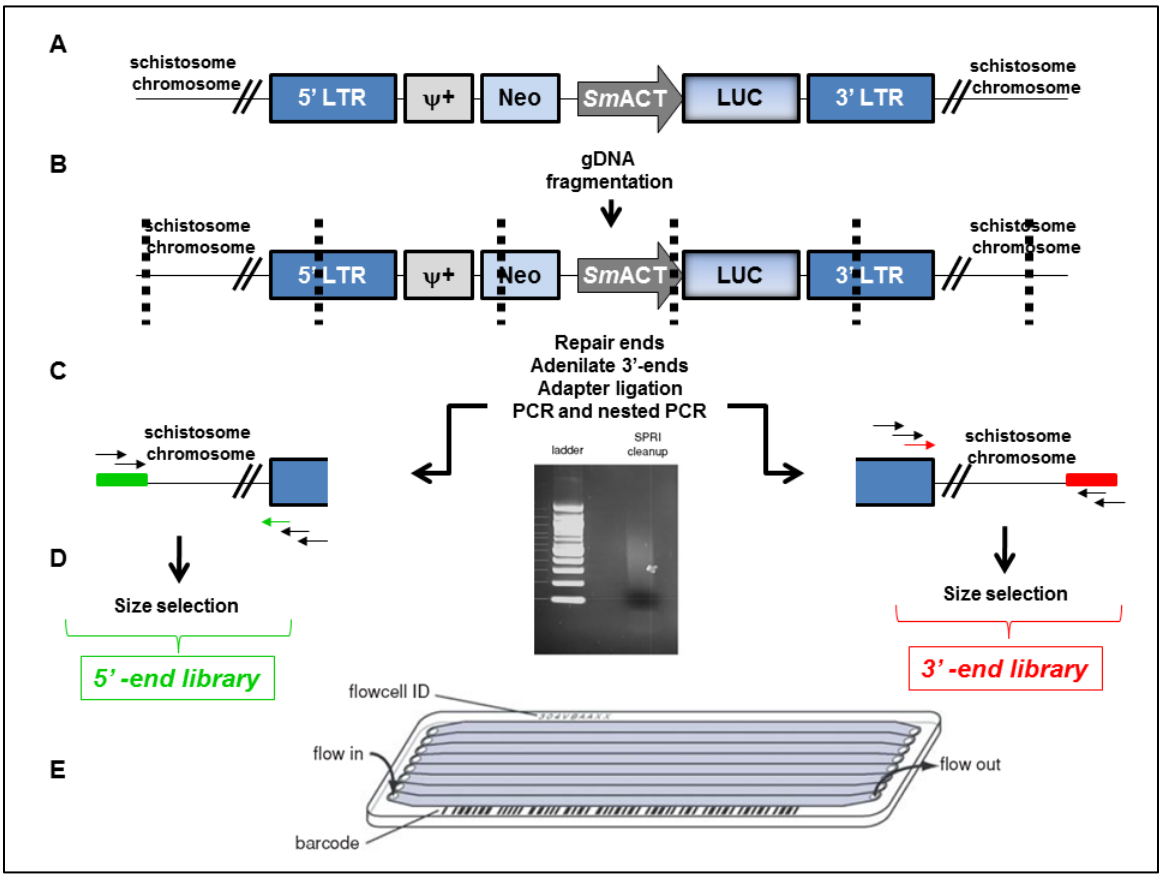

Figura 11. Diagrama de la estrategia seguida para la preparación de la bibliotecas de secuenciación Illumina.
A: Esquema del ADN aislado de parásitos transducidos con MLV, B: Fragmentación mecánica del ADN, C: Reparación de los extremos de los fragmentos, adenilación de los extremos 3', ligación de los adaptadores de Illumina, PCR y nested PCR utilizando cebadores dirigidos contra cada uno de los adaptadores y los extremos 5' y 3' del provirus, D: Selección de las bibliotecas de los extremos 5' y 3' por tamaño y purificación de la banda de amplificación del nested PCR, E: Siembra de las bibliotecas de los extremos 5' y 3' en la placa de secuenciación del Illumina.

Análisis bioinformático

Luego de una fase inicial de control de calidad (eliminación de las secuencias de los adaptadores y cebadores), se seleccionaron las lecturas de secuencia que contenían los extremos del provirus (las ultimas 7 pb de los extremos 5' y 3') y la secuencia adyacente fue mapeada sobre la base de datos genómica de *Schistosoma mansoni* (Berriman et al. 2009). Se consideraron integraciones genuinas aquellas secuencias que siguieron los siguientes criterios: (1) la secuencia Illumina comenzaba en el extremo LTR del provirus aceptando una diferencia de no más de 1 pb con la secuencia LTR original, (2) el resto de la secuencia adyacente al LTR mapeaba en el genoma de Schistosoma al menos en 40 pb, y (3) con una calidad de mapeo ≥ 30 (correspondiendo a 0.1% de tasa de error de alineamiento). Se descartaron secuencias que mapearon en regiones de baja complejidad en el genoma, y se categorizaron las integraciones comparándolas con la anotación del GeneDB en regiones no codificantes, regiones codificantes (exones), intrones y 5'UTRs. Dado que no hay una información disponible de las regiones 5'UTRs, asumimos como 5'UTR la secuencia comprendida entre el codón de iniciación de la traducccion del gen y 3000 bases corriente arriba.

B. Desarrollo de la transgénesis mediada por vectores retrovirales en *Fasciola hepatica*

Desenquiste in vitro de metacercarias y cultivo de juveniles recientemente desenquistados de F. hepatica (JRD)

La obtención de metacercarias de *Fasciola hepatica*, su desenquiste *in vitro* y el mantenimiento en cultivo de los juveniles recientemente desenquistados (JRD) se encuentran detallados en (Cancela *et al.* 2008; Rinaldi 2008; Rinaldi *et al.* 2008).

Virus de la leucemia murina modificado, vectores retrovirales y transducción de juveniles de F. hepatica

Los virus de la leucemia murina modificado (MLV) fueron producidos y cuantificados según los protocolos descritos más arriba. Veinticuatro horas luego de su recolección, los juveniles de *F. hepática* fueron expuestos al MLV ya sea por simple inoculación de las partículas virales en el medio de cultivo o mediante electroporación siguiendo el protocolo detallado en (Rinaldi *et al.* 2008). Al cabo de dos días en cultivo luego de la exposición de los parásitos al MLV, algunos de éstos fueron fijados para estudios de inmunolocalización de antígenos virales y otros fueron procesados para la extracción de ADN genómico y su análisis para la búsqueda de transgenes.

Inmunolocalización de antígenos virales en la superficie de juveniles transducidos con MLV

Los juveniles de *F. hepatica* cultivados en presencia de los viriones MLV modificados expresando VSVG en su superficie (ver más arriba) por 30 o 180 minutos, y controles no expuestos a virus, fueron lavados con PBS para remover las partículas virales no unidas al parásito y fijados con paraformaldehído. Siguiendo el protocolo descrito en (Kines *et al.* 2008), los parásitos fueron incubados con un anticuerpo primario anti-VSVG de conejo y uno secundario anti-IgG de conejo conjugado al fluorocromo Alexa Fluor 488. La señal fue monitorizada y registrada utilizando una cámara acoplada a un microscopio de fluorescencia.

Análisis de la presencia por PCR convencional y en tiempo real del transgen en juveniles de F. hepatica transducidos con MLV

La extracción de ADN y la evaluación del mismo mediante PCR convencional y en tiempo real se llevaron a cabo siguiendo el protocolo detallado en el artículo (Kines *et al.* 2010) presentado en el capítulo de Resultados y Discusión.

RESULTADOS

La mayor parte de los resultados correspondientes a las distintas secciones indicadas en Objetivos y Estrategia General, ya han sido publicados en revistas arbitradas internacionales, por lo que se incluyen estas publicaciones en las sub-secciones correspondientes. En un caso existe un manuscrito que está siendo considerado por los consejos editoriales de revistas internacionales arbitradas.

A. OPTIMIZACIÓN DE LA TRANSGÉNESIS EN *SCHISTOSOMA MANSONI*

A.1. Cuantificación de transgenes en parásitos transducidos con vectores retrovirales

ARTICULO: Eficiencia de transducción de transgenes en adultos de *Schistosoma mansoni* confirmada mediante PCR cuantitativo anclado a retroposones.

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(* Igual contribución al trabajo)

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Short technical report

Quantitative retrotransposon anchored PCR confirms transduction efficiency of transgenes in adult *Schistosoma mansoni*

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ABSTRACT

A quantitative retrotransposon anchored PCR (qRAP) that utilizes endogenous retrotransposons as a chromosomal anchor was developed to investigate integration of transgenes in *Schistosoma mansoni*. The qRAP technique, which builds on earlier techniques, (i) *Alu*-PCR which has been used to quantify lentiviral (HIV-1) proviral insertions in human chromosomes and (ii) a non-quantitative retrotransposon anchored PCR known to detect the presence of transgenes in the *S. mansoni* genome, was tested here in a model comparison of retrovirus-transduced adult schistosomes in which one group included intact worms, the other included fragments of adult worms. At the outset, after transducing intact and viable fragments of schistosomes with reporter RNAs, we observed more reporter activity in fragments of worms than in intact worms. We considered this simply reflects the increased surface area in fragments compared to intact worms exposed to the exogenous reporter genes. Subsequently, intact worms and worm fragments were transduced with pseudotyped virions. Transgene integration events in genomic DNA extracted from the virion-exposed worms and worm fragments were quantified by the qRAP, which revealed that fragmenting adult schistosomes resulted in increased density of proviral integrations. The qRAP findings confirmed the likely value of this qRAP technique for quantification of transgenes integrated in schistosome chromosomes. Last, considering the absence of schistosome cell or tissue lines, primary culture of fragmented worms offers an opportunity to optimize transgenesis, and other functional genomic approaches.

1. Introduction

The genome sequences of two of the three major species of schistosomes are now available [1,2], and transcriptomic data on other trematodes are rapidly increasing [3,4]. The identification and validation of putative gene function requires functional genomics methods and tools that are now in development, e.g., [5–10]. RNA interference (RNAi) has been employed to determine the function and importance of a number of schistosome genes (e.g.[11–13]), and its deployment is expected to expand [14]. Whereas RNAi provides insights, transgenesis approaches may provide a flexible framework for both reverse and forward genetics in schistosomes and other trematodes. Recently, it has been demonstrated that vesicular stomatitis virus glycoprotein pseudotyped murine leukemia virus virions (VSVG-MLV) can be employed to transduce the surface of several developmental stages of both *Schistosoma mansoni* [15–17] and *Schistosoma japonicum* [18], including eggs, sporocysts, schistosomules and adult worms. Furthermore, vector based RNAi was employed recently to investigate the importance of schistosome genes; *in vitro* transcription of short hairpin RNAs specific for the gene encoding a papain-like cathepsin B of *S. mansoni* rapidly depleted adult worms of target protease [19].

Because of the nature of the gammaretroviral life cycle, the proviral genome of MLV integrates into the genome of the transduced cell [20]. Studies with VSVG-MLV have confirmed that proviral sequences integrate in the chromosomes of *S. mansoni* [16]. A potential functional genomics application of this ability of MLV to integrate in the schistosome genome is for insertional mutagenesis screen of schistosomes, now feasible given that draft genome sequences for *S. mansoni* and *S. japonicum* are available. Langridge and colleagues [21] recently demonstrated that power of insertional mutagenesis using a bacterium-transposon model, and indeed were able to characterize *Salmonella* Typhi genes that were essential for this enteric pathogen. Whereas each developmental stage of the schistosome life cycle might provide particular advantages to be targeted for introduction of transgenes (see [8,17,22]),

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the adult worms are readily and reliably obtained from experimentally infected rodents, can be easily maintained in culture, and the females continue to release viable eggs for several days after perfusion from mice [23]. These characteristics make the adult developmental stage an attractive target for transgenesis. However, since replication-deficient retroviral systems can only integrate into the chromosomes of the cell to which the provirus attaches, only the surface and gut are readily targeted in cultured schistosomes.

In this study we analyzed whether fragmenting the adult worm and maintaining the fragments in culture (a procedure that reduces the large ratio of surface area to body mass in schistosome adults) might result in improved transduction rates. A quantitative retrotransposon anchored PCR (qRAP) that utilizes endogenous retrotransposons as a chromosomal anchor was developed to investigate integration of the transgenes. Using the qRAP, we confirmed increased density of transgenes in cultured fragments of adult schistosomes, and more importantly confirmed the utility of this anchored PCR to quantify transgene transduction.

2. Materials and methods

2.1. Parasites

Mixed sex adults of *S. mansoni* were perfused from experimentally infected mice six to nine weeks after infection and maintained in culture [22].

2.2. Synthesis and delivery of luciferase mRNA

To synthesize firefly luciferase mRNAs (mLuc), DNA templates were amplified by PCR from plasmid pGL3-Basic (Promega, Madison, WI) templates, as described [5]. *In vitro* transcriptions of capped RNAs from template PCR products were accomplished using the mMessage mMachine T7 Ultra kit (Ambion, Austin, TX). mRNAs were precipitated with ammonium acetate, dissolved in nuclease-free water and concentration determined with a spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE). Mixed sex adult worms were removed from culture [22], washed five times in schistosomule wash medium (Dulbecco's modified Eagle's medium (DMEM) with 200 U/ml penicillin G sulfate, 200 mg/ml streptomycin sulfate, 500 ng/ml amphotericin B, 10 mM HEPES) and sliced in two, three or more pieces, as indicated, using a sterile scalpel blade. Thereafter, the intact worms and the worm fragments were subjected to square wave electroporation (250 V, 30 ms) in 4 mm gap BTX cuvettes containing 6 μg of firefly luciferase mRNA in 100 μl of schistosomule wash medium. After electroporation, intact worms and worm fragments were transferred to DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U of penicillin and streptomycin (Invitrogen, Carlsbad, CA), pre-warmed to 37 °C [22]. Three hours after electroporation, the worms were washed well with schistosomule wash medium, and then stored as wet pellets at −80 °C.

2.3. Luciferase activity assay

Luciferase activity in extracts of these schistosomes was determined using Promega's luciferase assay reagent system in a Sirius luminometer (Berthold, Pforzheim, Germany), as described [7]. In brief, pellets of parasites were subjected to sonication (3 × 5 s bursts, output cycle 4, Misonix Sonicator 3000, Newtown, CT 06470) in 300 μl 1× CCLR lysis buffer (Promega). The sonicate was cleared by centrifugation at 14,000 rpm at 4 °C for 15 min (Eppendorf model 5810 centrifuge), and activity in the supernatant containing the soluble fraction determined. Aliquots of 100 μl of the soluble fraction were dispensed into100 μl luciferin substrate (Promega) at 23 C, mixed, and the relative light units (RLUs) were determined in the luminometer 10 s later. Duplicate samples were measured, with results presented as the average of the duplicate readings per mg of soluble fluke protein. Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Recombinant luciferase (Promega) was included as a positive control.

2.4. Exposure of fragmented worms to Cy3-labeled siRNA

S. mansoni mixed sex adults were fragmented and subjected to square wave electroporation in the presence of Cy3-labeled siRNAs (Silencer Cy3-Labeled Negative Control siRNA, catalog AM4621, Ambion, Austin, TX) at 3.6 μM (50 ng/μl) following the conditions described above. Immediately after electroporation parasites were transferred into complete DMEM at 37 °C. Four hours after exposure to Cy3-siRNA, worms were washed in culture medium five times in order to remove the unincorporated Cy3-labeled siRNAs. Thereafter, they were observed under bright and fluorescent light (see below) using a Zeiss Axio Observer A.1 inverted microscope fitted with a digital camera (AxioCam ICc3, Zeiss). Manipulation of digital images was undertaken with the AxioVision release 4.6.3 software (Zeiss).

2.5. Transduction of schistosomes with pseudotyped murine leukemia retrovirus (VSVG-MLV)

VSVG-pseudotyped virions were produced in GP2-293 cells transfected with plasmid constructs pLNHX-SmAct-Luc and pVSVG [15]. Viral titers were determined using two complementary approaches; a functional (biological) assay using target NIH-3T3 mouse fibroblast cells cultured in the presence of geneticin [15] and second by real time PCR (Retro-X^{TM} qRT-PCR Titration Kit, Clontech). Intact or fragmented worms were cultured in 24 well plates, in 200 μl of medium plus 200 μl of virions (VSVG-MLV) at 4×10^5 colony forming units (cfu)/ml in the presence of 8 μg/ml polybrene (Sigma–Aldrich, St. Louis, MO). The same preparation of virus was used to estimate the viral titer by real time PCR resulting in a viral titer of 7.03×10^7 copies/ml. The worms were washed 18 h later and cultured for a further 24 h, after which genomic DNAs (gDNAs) were extracted from the worms. The density of integrated proviral luciferase transgenes investigated in the gDNAs was investigated by quantitative, anchored PCR (below).

2.6. Quantitative-retrotransposon anchored PCR (qRAP)

Based on the *Alu*-PCR approach used to quantify the copy number of integrated HIV-1 provirus in the genome of human cells [24], and on a chromosomal anchored PCR technique we have used previously to identify transposon and proviral transgenes in the genome of *S. mansoni* [6,16], we developed a quantitative anchored PCR-based approach (qRAP), to investigate retrovirus integrations into the schistosome genome. In brief, qRAP includes two consecutive PCRs (Fig. 1). The first, retrotransposon anchored PCR (RAP), consists in a multiplex PCR using a specific primer for the luciferase (*luc*) transgene from the donor pLNHX construct in tandem with primers specific for endogenous retrotransposons present at high copy number and interspersed throughout the genome of natural populations of *S. mansoni* [1,25]. Second, RAP products are used as template in a quantitative PCR, targeting the *luc* transgene [17]. The first RAP amplification was performed using 100 ng template gDNA from populations of MLV-transduced schistosomes or control gDNA from untreated worms, Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) and primers specific for the retrotransposons *SR1*, *SR2*, *fugitive* and *Boudicca* in combina-

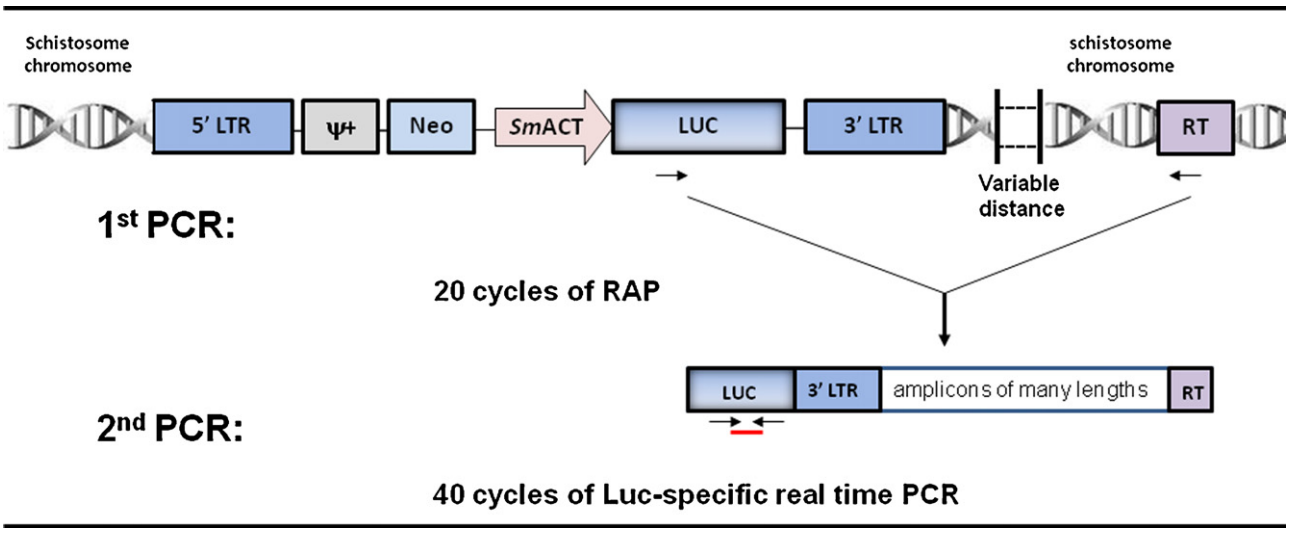

Fig. 1. Illustration of the quantitative Retrotransposon Anchored PCR (qRAP) approach. First PCR: 20 cycles of end-point PCR preamplification with primers that target endogenous mobile genetic elements and luciferase transgene sequences. Heterogeneous amplicons of variable length are expected. Second PCR: quantitative PCR to estimate the copy number of luciferase-specific sequences within the transduced schistosome genome. Quantification was undertaken using copy number standards, i.e. 10-fold serial dilutions of the luciferase encoding plasmid pGL3, after which copy number of luciferase transgene in schistosome genomic DNAs was calculated by interpolation from a standard curve. The qRAP was adapted from Ref. [24].

tion with the luciferase-specific primer in a 50 μl reaction. Four primer mixes were used: mix #1, *SR1*F (200 nM), *SR1*R (200 nM) and *luc* (400 nM); mix #2, *SR2*F (200 nM), *SR2*R (200 nM) and *luc* (400 nM); mix #3, *fugitive* F (200 nM), *fugitive* R (200 nM) and *luc* (400 nM); mix #4, *Boudicca* F (200 nM), SMα (200 nM), *luc* (400 nM) (Table S1).

Linear, one-way amplification was also evaluated by performing the preamplification PCR with the *luc* primer alone. RAP cycling conditions were 94 °C for 2 min followed by 20 cycles of 94 °C for 30 s, 57 °C for 30 s and 68 °C for 10 min, with a final extension at 68 °C for 10 min. RAP products were employed as template in a quantitative PCR targeting *luc* was performed as described [17]. Briefly, *luc* specific primers and TaqMan probe were designed with the assistance of Beacon Designer (Premier Biosoft International, Palo Alto, CA); sequences of the primers and probe: *luc* forward primer, 5′-TGC TCC AAC ACC CCA ACA TC-3′; reverse primer, 5′-ACT TGA CTG GCG ACG TAA TCC-3′; probe, 5′-/56-FAM/ACG CAG GTG TCG CAG GTC TTC C/3IABlk_FQ/-3′. The PCR efficiency for the luciferase primers/probe set was estimated by titration analysis [26] to be 97% (not shown).

Quantitative PCRs were performed in triplicate, using 96-well plates (Bio-Rad), with a denaturation step at 95 °C of 3 min followed by 40 cycles of 30 s at 95 °C and 30 s at 55 °C, using a real-time thermal cycler (iCycler, Bio-Rad) fitted with the Bio-Rad iQ5 detector. Reactions were carried out in 20 μl volumes with *luc* primer-probe sets, Perfecta qPCR FastMix, UNG (Quanta Bioscience, Gaithersburg, MD), and using as template, 5 μl of a 0.1 dilution of the RAP products (preamplification dilutions) or matched dilutions of non-preamplified samples, i.e., gDNA dilutions that were not amplified by RAP. Quantification was undertaken using copy number standards, i.e. 10-fold serial dilutions of the firefly luciferase encoding plasmid pGL3 Basic (Promega), from 1.93×10^3 copies to 1.93×10^{10} copies. Luciferase transgene copy number was estimated by interpolation of the sample PCR signals from a standard curve [26]. Transgene copy numbers from schistosomes exposed to virions are presented as fold-increase of RAP-preamplified copy number compared to the non-preamplified copy number.

3. Results

3.1. Reporter RNAs introduced into schistosomes

Fragmented and intact worms were transduced with mRNA encoding firefly luciferase, and luciferase activity measured three hours later (Fig. 2A). Luciferase activity was readily detected in extracts of intact adult worms. As illustrated (Fig. 2B), significantly higher luciferase activity was seen in extracts of worms that had been cut into two pieces and even higher activity seen in extracts of the worms chopped into three or more fragments (646, 1,066 and 2,039 RLUs/s/mg protein, respectively) ($P \leq 0.05$). In summary, the more fragmented the worms, the higher the luciferase activity.

To investigate whether higher luciferase activity measured in fragmented parasites was caused by increasing the surface area exposed to the reporter, we transduced fragmented parasites with Cy3-labeled siRNA, a fluorescent probe employed previously to demonstrate entry of macromolecules into other trematode developmental stages [7,17]. Four hours after electroporation, the worm pieces were washed and examined. As is shown in Fig. 3 bright foci of fluorescence were evident at or proximal to the cut surfaces of the worms indicating that Cy3-labeled siRNA was introduced into the worms not only through the intact tegument but apparently also through the lesions.

3.2. Retrotransposon anchored PCR quantifies transgene integrations

Increasing the surface area of transduced worms facilitated entry of reporter RNAs. Accordingly, we proceeded to transduce intact and fragmented schistosomes with VSVG-MLV virions. Integration events in gDNAs extracted from the worms were estimated by qRAP, using primer pairs targeting the luciferase transgene and endogenous mobile genetic elements that occur at high density in the *S. mansoni* genome [1,6,16]. With each of four separate anchored primer sets, targeting the *SR1*, *SR2*, *fugitive* or *Boudicca* (plus SMα) retrotransposons, similar trends were seen – fragmenting the schistosomes resulted in increased density of proviral integrations. As shown in Fig. 4A, left panel, the qPCR signal

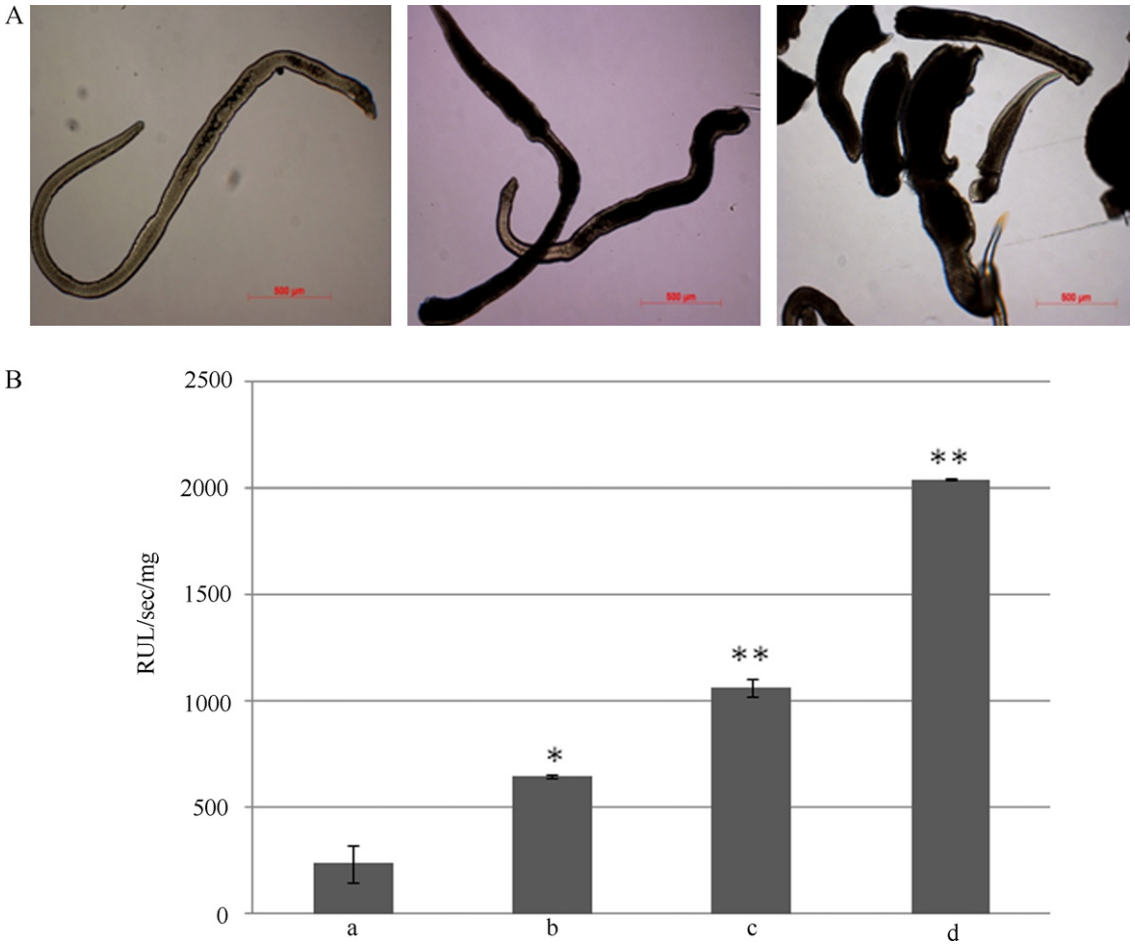

Fig. 2. Luciferase activity in adult *Schistosoma mansoni* worms and worm pieces following transduction with firefly luciferase mRNA. Panel A: representative images of an intact female adult (left panel), a female adult cut in two pieces (middle panel) and worms diced into three or more pieces (left panel). Scale bar, 500 μm. Panel B: Luciferase activity measured in extracts of adult worms 3 h after electroporation, (a) mock control adult worms treated with no molecule, (b) intact worms treated with mRNA, (c) adult worms cut in two pieces and treated with mRNA and (d) worms diced into three or more pieces and treated with mRNA. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

detected from fragmented parasites amplified with the *SR1* primer mix crossed the threshold cycle about four cycles earlier than for intact parasites. The signal from control, untreated worms did not reach the threshold before 40 cycles. Similar outcomes were seen with the *SR2*, *fugitive* and *Boudicca*/SMα primer mixes (not shown). Significant difference in threshold cycle was observed for RAP-preamplified compared with non-preamplified templates, i.e. gDNA that was amplified only by qPCR (Fig. 4A, centre panel). When one-way amplification was monitored by performing the first PCR with only the *luc* primer, again a significant difference in threshold cycle was detected for RAP-preamplified compared with either preamplified with only the *luc* primer or with the non-preamplified templates (Fig. 4A, right panel).

Given the lack of an accurate quantitative standard curve in our model, as in the *Alu*-PCR of O'Doherty et al. [24] who developed an integration standard $CD4^{+}$ T-lymphoblastoid cell line in which the copy number of integrated HIV-1 provirus was known, with our qRAP we cannot predict the absolute transgene copy number per nanogram of virion-transduced gDNA. However, we were able to predict the absolute transgene copy number from samples that were not RAP-preamplified. Consequently, we have estimated fold-increase of transgene copies between RAP-preamplified and non-preamplified samples. As shown in Fig. 4B, the transgene copy number fold-increase estimated from parasites that had been cut into pieces was significantly higher than in intact worms. Specifically, the transgene copy number fold-increase between the RAP-preamplified samples and matched non-preamplified samples was 5–7-fold (depending on the anchoring primer – *SR1*, *SR2*, etc.) in intact worms and ~16–24 fold in the fragmented worms.

4. Discussion

We observed increased incorporation of exogenous reporter RNA into fragmented compared to intact schistosomes. Further, we estimated the density of transgene chromosomal integrations in the treated worm population using a quantitative retrotransposon anchored PCR approach. This procedure that we termed qRAP is an adaptation of previous methods [6,16,24], and involves the amplification of integration events by a multiplex PCR using a transgene primer and a set of primers directed to retrotransposons widely dispersed in the schistosome genome, followed by a quantitative PCR determination of the amount of transgene copies against a standard. We anticipate qRAP can now be employed to quantify transgene copy number in transgenic schistosomes and other helminths.

Although the qRAP technique does not provide absolute copy number per genome, it is useful for comparing integration events between treatment conditions. Here, significant differences were observed between the transgene copy number fold-increase in parasites that had been diced into pieces than in the intact worms: ~6-fold in intact worms and ~20-fold for diced worms (Fig. 4B). Depending on the anchoring primer – *SR1*, *SR2*, *fugitive* or *Boudicca*/SMα the transgene copy number fold-increase between

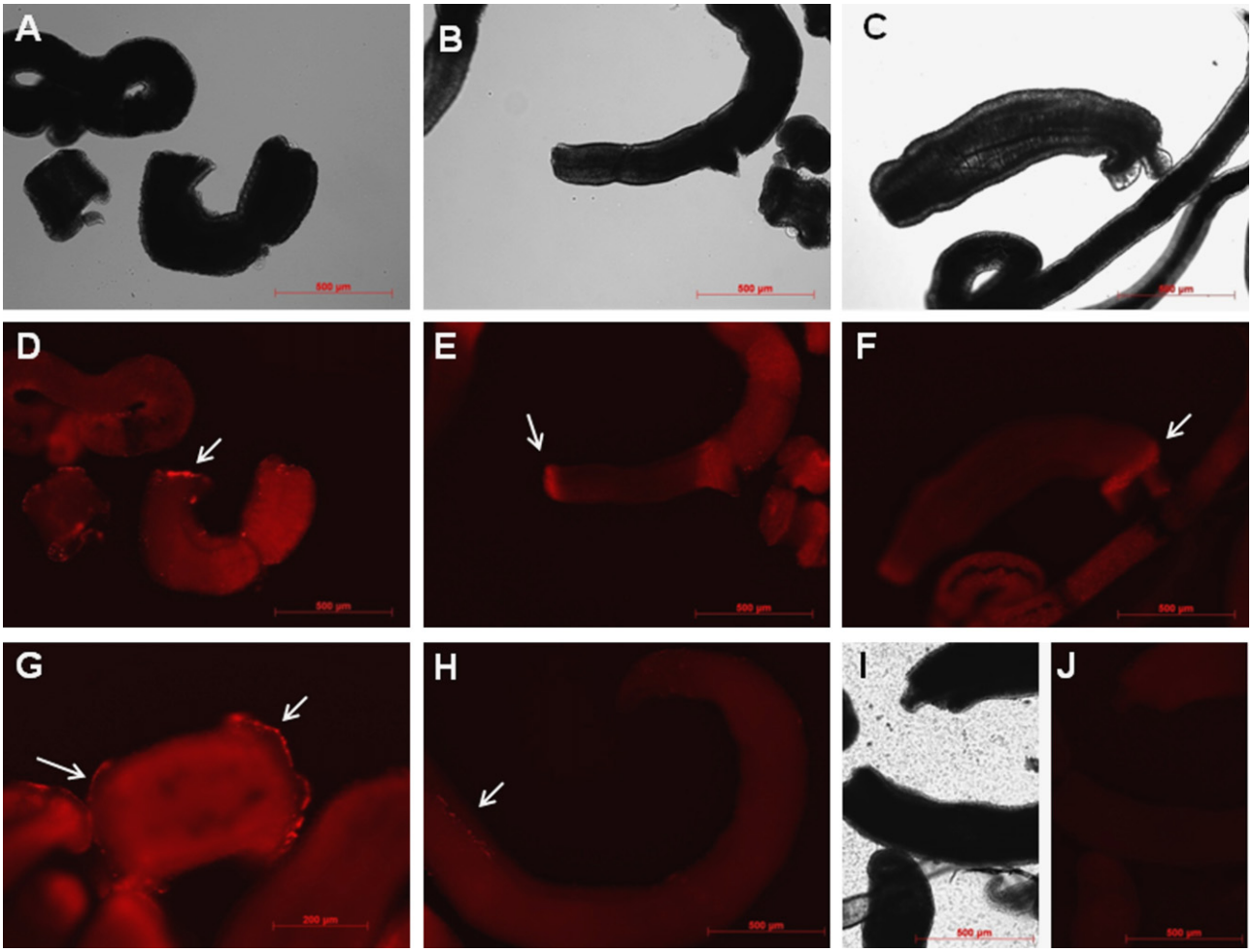

Fig. 3. Fluorescent Cy3-labeled short interfering RNA enters fragmented adults of *Schistosoma mansoni*. Fragmented schistosome adults four hours after electroporation in 50 ng/μl Cy3-siRNA; (A–C) bright and (D–F) fluorescent fields of representative images of fragmented worms, (G) high magnification image of a representative fragmented worm with fluorescent foci in sliced region, (H) representative image of an intact worm showing fluorescent foci in the tegument, (I) bright and (J) fluorescent fields showing control, untreated worms. White arrows indicate fluorescent foci. Scale bar 500 μm in A–F and H–J, and 200 μm in G.

the RAP-preamplified samples and matched non-preamplified samples range from 5 to 7 in intact worms and from 16 to 24 in sliced parasites, e.g. the highest fold-increase was observed in diced parasites when *SR2* was used. This could reflect the variation in abundance among the target retrotransposons in the *S. mansoni* genome [1]. The fold increases between RAP-preamplified and non-preamplified samples were lower than those reported by O'Doherty and colleagues for HIV-1 using quantitative *Alu*-PCR [24]. However, whereas they observed a ~4,000-fold difference with a chronic HIV-infected cell line, they reported a much smaller difference (~200-fold) between pre-amplified and non-preamplified gDNA from an acute *in vitro* infection. In the present study, we harvested worms one day after virion exposure, conditions more similar to the acute rather than chronically infected cells of O'Doherty et al. [24]. In addition, there are additional reasons why the qRAP may not be as efficient as *Alu*-PCR including (1) differences in biological characteristics of the target retrovirus, HIV-1 versus MLV, (2) densities of *Alu* in human chromosomes (>1.1 million copies) and the endogenous schistosome retrotransposons (<10,000 copies), (3) relative frequency of truncation phenomena of MLV proviral genome in the schistosome genome [16], and (4) distance of transgene primer from the 3′-LTR (Fig. 1).

Despite these potential impediments, the present findings confirmed that qRAP facilitates comparison of integration outcomes between treatment conditions. Although an accurate standard curve would enhance estimation of absolute transgene copy number in transgenic schistosomes, qRAP represents the first approach that allows relative quantification of integration events in schistosomes. We anticipate that qRAP will find utility in evaluating transduction/integration efficiency of retroviruses or other integrative vectors, such as transposons or retrotransposons. We predict that functional genomics for schistosome will be enhanced by transgenesis studies utilizing VSVG-MLV, as several recent reports have indicated [16,17,19]. As methods both to quantify and to increase the genomic density of integrations, the techniques described here should enhance the prospects of progress with functional genomics with MLV-based transgenesis of schistosomes. In addition, increasing the density of retrovirus integration events not only would allow to identify preferential target sites in schistosome chromosomes, but also would facilitate insertional mutagenesis screens and in development of gain-of-function studies, for example with selection for drug resistance, reporter gene activity, and so forth.

Acknowledgements

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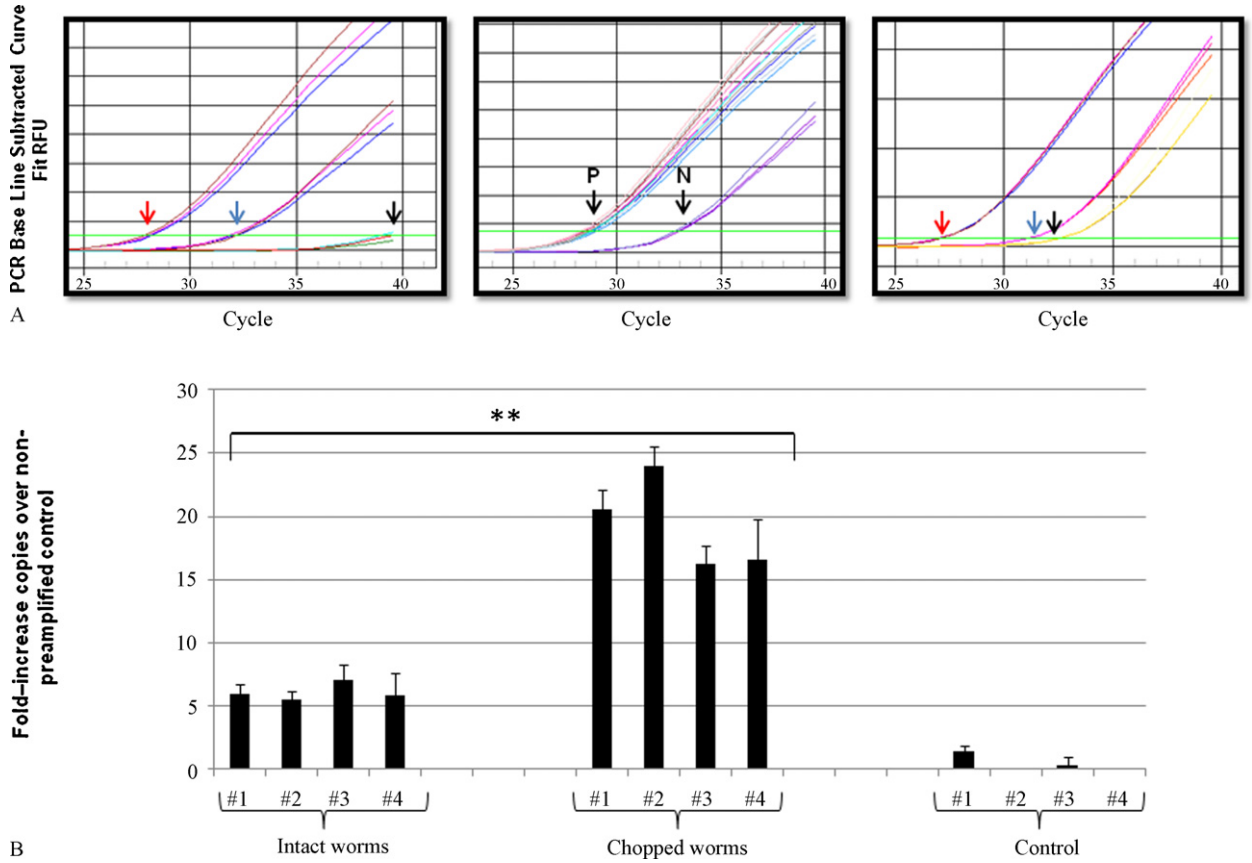

Fig. 4. Relative transgene copy number estimated by qRAP in the genome of virus exposed adults of *Schistosoma mansoni*. (A) Left panel. Amplification plots observed in preamplified samples using SR1 primer mixes. Arrows point threshold cycle of the indicate sample, red arrow: MLV-transduced fragmented worms, blue arrow: MLV-transduced intact worms, black arrow: control untreated worms. Centre panel. Amplification plots observed in preamplified (P) and non-preamplified (N) MLV-transduced fragmented worms using all primer mixes. Arrows point threshold cycle of the indicate sample. Right panel. Amplification plots observed in MLV-transduced fragmented worms in preamplified template using SR2 primer mix (red arrow), in preamplified template using only the luc primer (blue arrow), and in non-preamplified template (black arrow). Arrows indicate the threshold cycle. (B) Transgene copy numbers in virions exposed schistosomes shown as fold-increase of preamplified copy number compared to the non-preamplified copy number in each sample. #1: *SR1* primer mix, #2: *SR2* primer mix, #3: *fugitive* primer mix, #4: *Boudicca*/SMα. ANOVA $^{**}P \leq 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2011.01.007.

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A.2. Evaluación de la integración genómica de los transgenes

Con la intensión de identificar a escala genómica los sitios preferenciales de integración del retrovirus de la leucemia murina (MLV) en *Schistosoma mansoni*, empleamos secuenciación con tecnología de la nueva generación (Illumina).

Transducimos gusanos adultos y schistosomulas con MLV y luego de 48 horas de cultivo aislamos el ADN genómico. Por otro lado, incubamos huevos del parásito en presencia de MLV, los eclosionamos, utilizamos los miracidios resultantes para infectar caracoles y se extrajo ADN genómico de las cercarias emitidas por dichos caracoles. Denominamos transgénesis somática a la resultante de la incorporación de transgenes a las células somáticas de schistosomulas o gusanos adultos. A su vez la denominada transgénesis germinal resulta de la transducción de células de la línea germinal con la potencialidad de transmitir el transgen a la siguiente generación. El ADN extraído de los estadios de desarrollo anteriormente mencionados se procesó para la secuenciación mediante Illumina, y los eventos de integración del provirus detectados fueron mapeados sobre el genoma de *Schistosoma mansoni* (Berriman *et al.* 2009).

La **figura 12** muestra la distribución en los cromosomas de *S. mansoni* de 1248 sitios de integración del provirus analizados. En estos resultados primarios, pero promisorios, se detectó una integración aleatoria del virus en el genoma del parásito, sin ningún sitio preferencial evidente. Este patrón observado es diferente de los descripto para MLV en células de mamífero donde el provirus se integra preferencialmente en las regiones 5' de los genes (Lewinski *et al.* 2006).

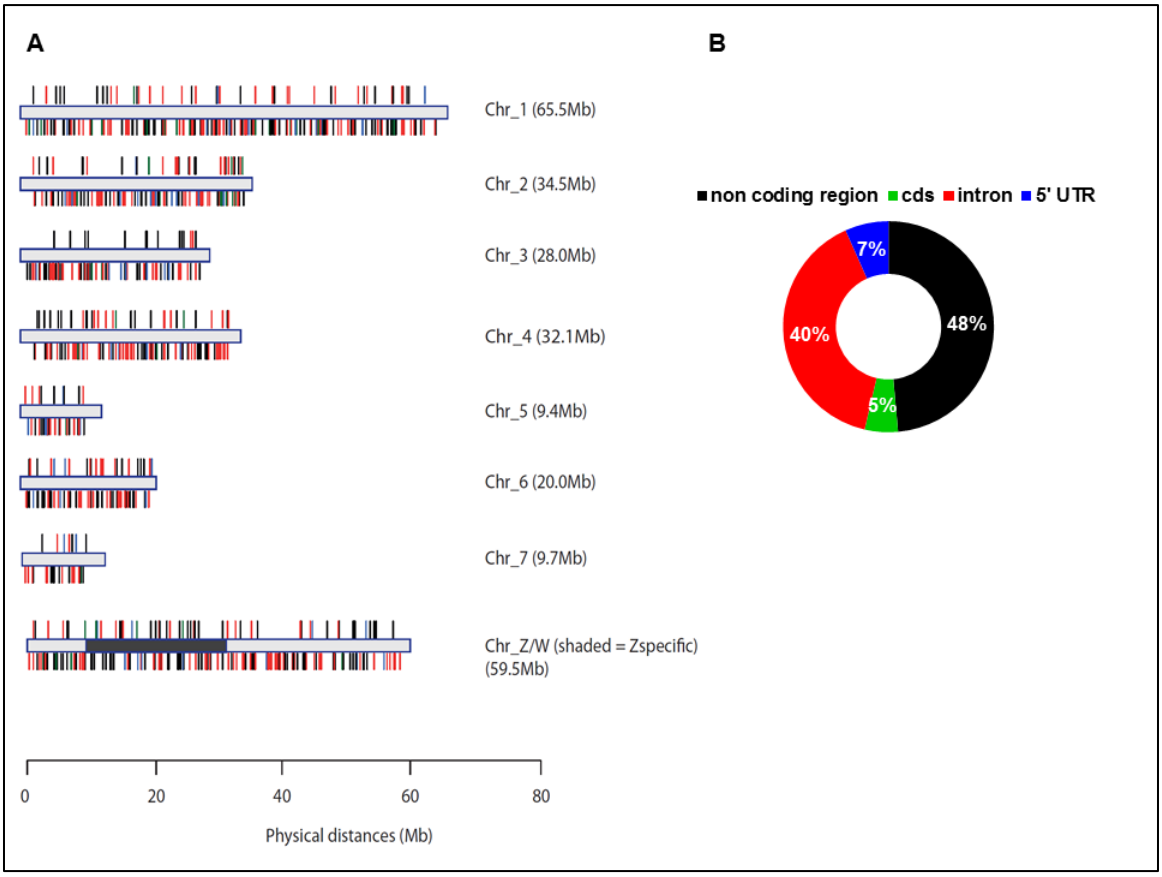

Figura 12. Sitios de integración del provirus de la leucemia murina (MLV) en el genoma de Schistosomas transducidos.

Panel A: Los cromosomas de Schistosoma mansoni son representados como barras horizontales. Los sitios de integración de MLV mostrados sobre el borde superior de los cromosomas fueron detectados en el ADN de adultos y schistosomulas transducidas (Transgénesis somática). Los sitios de integración de MLV mostrados en el borde inferior de los cromosomas fueron detectados en el ADN de cercarias emitidas por caracoles infectados con miracidios de huevos transducidos con MLV (Transgénesis de la línea germinal).La escala en la parte inferior señala las distancias físicas del mapa.

Panel B: Gráfico de torta mostrando los porcentajes de sitios de integración distribuidos según las regiones del genoma indicadas; no codificantes, codificantes (cds), intrones y 5'UTRs.

A.3. Selección de organismos transgénicos

ARTICULO: Un marcador de selección para la transgénesis en *Schistosomas*

Gabriel Rinaldi, Sutas Suttiprapa, Jose F. Tort, Anne E. Folley, Danielle E. Skinner, Paul J. Brindley

International Journal for Parasitology (submitted)

An antibiotic selection marker for schistosome transgenesis

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Abstract Drug selection is widely used in transgene studies of microbial pathogens, mammalian cell and plant cell lines. Drug selection of transgenic schistosomes would be desirable to provide a means to enrich for populations of transgenic worms. We adapted murine leukemia retrovirus (MLV) vectors - widely used in human gene therapy research - to transduce schistosomes, leading to integration of transgenes into the genome of the blood fluke. A dose-response kill curve and lethal G418 (geneticin) concentrations were established: 125 to 1,000 µg/ml G418 were progressively more toxic for schistosomules of *Schistosoma mansoni* with toxicity increasing with antibiotic concentration and with duration of exposure. By day 6 of exposure to ≥500 µg/ml, significantly fewer worms survived when compared to non-exposed controls, and by day 8, significantly fewer worms survived than controls at ≥250 µg/ml G418. When schistosomules were transduced with MLV encoding the *neoR* transgene and cultured in media containing G418, the *neoR* transgene rescued transgenic schistosomules from the antibiotic; by day 4 in 1000 µg/ml and by day 8 in 500 µg/ml G418, significantly more transgenic worms survived the toxic effects of the antibiotic. More copies of *neoR* were detected per nanogram of genomic DNA from populations of transgenic schistosomes cultured in G418 than from transgenic schistosomes cultured without G418. This trend was G418 dose dependent, demonstrating enrichment of transgenic worms from among the schistosomules exposed to virions. Furthermore, higher expression of *neoR* was detected in transgenic schistosomes cultured in the presence of G418 than in transgenic worms cultured without antibiotic. The availability of antibiotic selection can be expected to enhance research progress on helminth parasites responsible for major neglected tropical diseases.

Keywords: Schistosome; transgene; antibiotic selection; neomycin; G418; geneticin; retrovirus; neglected tropical diseases; functional genomics; *neoR*

1. Introduction

Schistosomiasis is considered the most important of the helminth diseases in terms of morbidity and mortality with more than 200 million infected people and a further 800 million at risk. Treatment and control of this neglected tropical disease relies on a single drug, praziquantel. New interventions, including vaccines, drugs and diagnostics, are needed as a global health priority (Hotez et al., 2008; Brindley et al., 2009). 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 Sequencing and and Functional Analysis Consortium, 2009; Berriman et al., 2009). Analysis of target genes to underpin new interventions for schistosomiasis would be aided by functional genomics to validate the essentiality of genes to be targeted with novel drugs or vaccines. For *Caenorhabditis elegans*, methods for genetic manipulations are well advanced. In noteworthy recent progress on the technical toolkit for *C. elegans*, drug selection with antibiotics, neomycin and puromycin, has been demonstrated utilizing resistance genes perpetuated in the transgenic worms as extrachromosomal arrays (Giordano-Santini et al., 2010; Semple et al., 2010). A molecular toolkit would also enhance our capacity to perform genetic manipulations of schistosomes and other parasitic helminths, and recently there has been progress in this endeavor (Grevelding, 2006; Castelletto et al., 2009; Suttiprapa et al., 2011a, b). For example, murine leukemia retrovirus (MLV) vectors - widely used in human gene therapy research – have been adapted to transduce schistosomes, leading to chromosomal integration of reporter transgenes and transgene expression (Kines et al., 2008). As noted in regard to advances with *C. elegans*, genetic selection systems using antibiotics in combination with antibiotic resistance genes are a mainstay of molecular genetics research (Chamberlin, 2010). These flexible tools allow stringent,

conditional selection of experimentally manipulated individuals. Such a system might also facilitate rapid progress with functional genomics of schistosomes.

Here we investigated drug selection of transgenic schistosomes in order to provide a means to enrich for populations of transgenic worms in virion-exposed parasites. We determined that schistosomes were sensitive in culture to geneticin (aka G418), at doses similar for this aminoglycoside antibiotic to those reported for other eukaryotes including mammalian cell lines and free living nematodes (Yallop and Svendsen, 2001; Vakulenko and Mobashery, 2003; Giordano-Santini et al., 2010). Schistosomes that were transduced by retrovirus encoding *neoR*, the gene encoding resistance to neomycin, were rescued in culture from G418 toxicity, and the retroviral transgene copy number was enriched in comparison to transgenic schistosomes cultured in the absence of the antibiotic.

2. Materials and Methods

2.1 Schistosomes

Biomphalaria glabrata snails infected with *S. mansoni* were supplied by Dr. Fred Lewis, Biomedical Research Institute, Rockville, MD. Schistosomules were obtained from cercariae released from the snails. Cercariae were concentrated by centrifugation (980 g/15 min) and washed in Dulbecco's modified Eagle's medium supplemented with 200 units/ml of penicillin, 200 µg/ml of streptomycin, 500 ng/ml of amphotericin B and 10 mM HEPES (wash medium). Cercarial tails were removed by 20 passes through a 22G emulsifying needle, after which schistosomule bodies were separated from tails by Percoll gradient centrifugation (Lazdins et al., 1982). Schistosomula were cultured at 37°C under 5% CO_2 in Basch's medium (Mann et al., 2010).

2.2 Establishing a G418/geneticin dose response curve for schistosomules

Schistosomules were transferred to 8 µm mesh polyethylene terephthalate insert membranes and holders (BD Biosciences) inserted into 24-well tissue culture plates. Schistosomules, 500-1,000 per well, were cultured in 125, 250, 500 or 1000 µg/ml G418 (Geneticin, Invitrogen) or without G418 in Basch's medium for 10 days. Media were changed every second day. In some cases, the detergent Triton X-100 was included at 0.1% or 0.5% (Semple et al., 2010).

2.3 Assessment of viability of schistosomules

Viability of schistosomules was monitored and scored visually. To accomplish this, four or more micrographs of the schistosomules were taken on days 2, 4, 6, 8 and 10 of culture of each treatment condition using a Zeiss Axio Observer A.1 inverted microscope fitted with a 10 X magnification objective lens and a digital camera (AxioCam ICc3, Zeiss). Manipulation of digital images was undertaken with the AxioVision release 4.6.3 software (Zeiss). Each of the micrographs for each G418 concentration recorded non-overlapping regions of the culture plates.

To score the effect of G418 on cultured schistosomes, 150 to 200 schistosomules for each treatment and time point group were scored using non-overlapping micrographs. Schistosomules were scored as either - live or dead - based on appearance (Clegg and Smithers, 1972; Cottrell et al., 1989). Figure 1 presents representative images of live and dead schistosomules. Live worms were defined as light or lighter in color, usually with obvious internal organs including the nascent gut, and frequently with elongate, vermiform appearance (Figure 1, panels A, B). By contrast, dead schistosomules were defined as dark colored worms (brown or black), with a

granular appearance and often with more compact, rounded in appearance rather than elongate (Figure 1, panel C) (Cottrell et al., 1989). In some of experiments with G418, aliquots of schistosomules were removed from the culture and incubated in the vital dye Trypan blue (Invitrogen) at 0.1% in PBS for 10 minutes at 37˚C, 5% CO_2, after which they were scored as dead if stained blue or live if not stained and/or stained weakly and still moving (Figure 1C and S1) (Cottrell et al., 1989; Gold, 1997). In like fashion, in some experiments, aliquots of schistosomules were removed from the culture and incubated in the fluorophores fluorescein diacetate (FDA), which stains live schistosomules, and propidium iodide (PI), which stains dead schistosomules, using methods reported by Peak et al. (2010). Assessing viability by (i) visual monitoring of standard cultures, (ii) Trypan blue, or (iii) differential staining by FDA/PI of aliquots of cultured schistosomules all gave similar results (Figures 1, S1). Consequently, we employed the visual inspection since this procedure allowed maintenance in culture of the entire populations, which could be monitored repeatedly by capturing several micrographs (10X objective) of non-overlapping fields every two days.

2.4 Transduction of schistosomules with pseudotyped virions

Vesicular stomatitis virus glycoprotein (VSVG)-pseudotyped murine leukemia virus (MLV) virions were produced in GP2-293 cells (Kines et al., 2008), using pLNHX_SmAct-Luc or pLNHXΔD70 (Kines et al., 2008; Suttiprapa et al., 2011b). Viral titers were determined using two complementary approaches, a functional (biological) and second a real time PCR (Retro-X^{TM} qRT-PCR Titration Kit, Clontech) (Tavoloni, 1997; Mann et al., 2011; Rinaldi et al., 2011). Two days after transformation from cercariae, schistosomules were transduced with virions. Briefly, ~10,000 schistosomules were exposed to virions in one ml of medium, 8 µg/ml

polybrene (Sigma-Aldrich); two virion titers were employed, 8 x 10^5 colony forming units (cfu) (~10^8 virions estimated by qPCR) and 2.4 x10^6 cfu (~10^9 virions). Schistosomules exposed to polybrene but without virions were included as controls. Schistosomes were incubated with the virions for 18 h at 37ºC, 5% CO_2, washed, divided in groups and transferred into media containing G418 at 0, 125, 250, 500 or 1000 µg/ml. Media including G418 were replaced every second day for 10 days. Micrographs of the schistosomes were collected on days 1, 4, 6, 8 and 10. In addition, aliquots of schistosomules were removed at days 6 and 10 and stored as wet pellets at -80°C for analysis of transgene copy number and expression.

2.5 Estimation of the transgene copy number

Genomic DNAs (gDNAs) were extracted from virion transduced and control schistosomules and concentration determined by spectrophotometer. Quantitative PCRs were performed using TaqMan probes and primers specific for neomycin phosphotransferase II (*neoR*) (forward primer, 5'-GGA GAG GCT ATT CGG CTA TGA C-3'; reverse primer, 5'-CGG ACA GGT CGG TCT TGA C-3'; probe, 5'-/56-FAM/ CTG CTC TGA TGC CGC CGT GTT CCG /3IABIk_FQ/-3'). Reactions were performed using 200 ng of template gDNA in 20 µl of Perfecta qPCR FastMix, UNG (Quanta Bioscience) and primer-probe set. Quantitative PCRs were performed in triplicate, with a denaturation step at 95°C of 3 min followed by 40 cycles of 30 sec at 95°C and 30 sec at 55°C, in thermal cycler (iCycler, Bio-Rad) fitted with a real time detector (iQ5, Bio-Rad). Absolute quantification was undertaken using a standard curve with serial dilutions of plasmid pLNHX (Kines et al., 2010), from 9.9 x 10^5 copies to 9.9 x 10^9 copies. Copy number of diluted plasmid was established through the relationship between the molecular mass of pLNHX and the Avogadro constant. Absolute copy numbers of the *neoR* transgene per

ng of schistosome gDNA were determined by interpolation of the sample PCR signals from a standard curve (see Ginzinger, 2002). Results were plotted as absolute *neoR* copy number per ng of gDNA (Kines et al., 2010).

2.6 Analysis of transgene expression

Total RNA was extracted from virion-transduced and control schistosomules using the RNAqueous-4PCR kit (Ambion). Residual DNA was removed with DNase (TurboDNase, Ambion). cDNAs were synthesized from 60 ng total RNA using the iScriptTM cDNA Synthesis Kit (Bio-Rad, Hercules, CA). *NeoR* gene expression was investigated by end-point RT-PCR and by quantitative RT-PCR (qRT-PCR). End-point RT-PCR was performed using *neo* gene specific primers (F: 5'-TGT GCT CGA CGT TGT CAC TGA A-3'; R:5'- ATG AAT CCA GAA AAG CGG CCA-3'); expected amplicons size, 383 bp. Expression of *S. mansoni* actin gene (GenBank U19945) was used as an internal control using the primers, F: 5'-CAG TGT TCC CTT CCA TCG TT-3'; R: 5'-GGA CAG GGT GTT CTT CTG GA-3', expected amplicons size, 224 bp. PCR conditions included an initial denaturation at 94 ◦C for 30 s followed by 35 cycles of 60 s at 94 ◦C, 60 s at 52 ◦C, 90 s at 72 ◦C and a final extension at 72 ◦C for 10 min. Amplification products were separated by electrophoresis through 1% agarose, stained with ethidium bromide, visualized under UV illumination and digital images captured (Gene-Doc, Bio-Rad). qRT-PCR was performed using TaqMan probe and primers specific for *neoR* (above) and for *S. mansoni* glyceraldehyde 3-phosphate dehydrogenase (*Sm*GAPDH) (GenBank M92359) as follows: forward primer: 5'- TGT GAA AGA GAT CCA GCA AAC -3'; reverse primer: 5'- GAT ATT ACC TGA GCT TTA TCA ATG G -3'; probe: 5'-/56-FAM/ AAG ACT CCA GTA GAC TCA ACG ACA T /3IABIk_FQ/-3'. Quantitative PCRs were performed in triplicate using 96-well

plates (Bio-Rad), with denaturation at 95°C for 3 min followed by 40 cycles of 30 sec at 95°C and 30 sec at 55°C. The relative quantification assay $2^{-\Delta\Delta Ct}$ method was employed (Livak and Schmittgen, 2001), with results plotted as normalized fold expression of *neoR* gene relative to the reference gene *Sm*GAPDH, considering 1 = *neo* relative expression level measured in control schistosomules not exposed to virions (calibrator sample).

2.7 Statistical analysis

Bars on curves represent one standard deviation (SD) of the mean. Levels of statistical significance among treatments were determined using Analysis of Variance (ANOVA) and Student's *t*-test; *p*-values of ≤ 0.05 were considered to be significant.

3. Results

3.1 Schistosomes are sensitive to geneticin (G418)

To establish the sensitivity of *S. mansoni* to geneticin (G418), an antibiotic that inhibits protein synthesis (Colbere-Garapin et al., 1981), schistosomules were cultured in media with or without G418 at 125, 250, 500 and 1000 µg/ml (experimental design, Figure 2A). Micrographs of cultures were recorded every second day from days 2 to 10 from which viability of the schistosomules was determined by scoring the appearance of the worms, and percentages of live schistosomules were plotted over time. At day 2, no differences were evident among the groups. Significant differences were apparent by day 6 and onwards among schistosomules in 500 µg/ml, e.g., 75% and 45% of live parasites by days 6 and 8, respectively; 1000 µg/ml, 63 % and 25% of live parasites by days 6 and 8, respectively, and control schistosomules cultured without G418. By days 8 and 10, all the G418 treated groups of parasites showed significant mortality in

comparison to the controls (Figure 2B). Even at lower antibiotic concentration assayed, e.g. 250 µg/ ml G418, significantly fewer schistosomules survived at day 8 (61% live) and day 10 (46% live) compared with the control schistosomules cultured without G418 (>90% live). Figures 2C and S2 present representative images of schistosomules cultured in the absence or presence G418 at several drug concentrations. In addition to Figure 2, replicate dose response assays were carried out; similar findings were seen with each of the three replicates (Figure S2 and not shown).

Since detergents appear to facilitate access of the antibiotic puromycin in *C. elegans*, markedly improving the selection of drug resistance (Semple et al., 2010), we tested inclusion of Triton X-100 to the cultures. Schistosomules died soon (less than one day) after transfer to medium containing 0.1% or 0.5% Triton X-100, with or without G418 (Figure S2, panel F).

3.2 Transgenic schistosomes rescued by neoR transgene from G418 antibiotic

Given that G418/geneticin was lethal for cultured schistosomules under the conditions tested here, we investigated if VSVG-MLV transduced schistosomules might be rescued (selected for) in the presence of G418/ geneticin since neomycin phosphotransferase II (encoded by *neoR*) endows resistance to G418 as well as to neomycin and other aminoglycosides (Padilla and Burgos, 2010; Smith and Baker, 2002; Vakulenko and Mobashery, 2003). Schistosomules were transduced with virions generated from plasmid pLNHXΔD70 (Suttiprapa et al., 2011b). Control groups of schistosomes not exposed to virions were also included in the analysis.

Percentages of live schistosomules within each treatment group were plotted against time in culture in G418-containing media (Figure 3A): from day 4 or 6 onwards, differences in survival were evident among all groups ($F = 38.1$, $F\ crit = 2.8$, $p \leq 0.05$ within each time point).

Additionally, at each time point and G418 concentration, more MLV transduced parasites survived than did the cognate group (control) worms not transduced with virions. For example, by day 4 in 1,000 µg/ml G418, 80% of the VSVG-MLV transduced parasites were alive compared to 58% of the control parasites, $p \leq 0.05$. By day 8 in 500 µg/ml, 90% of the MLV transduced parasites survived whereas only 62% of the control schistosomules survived ($p \leq 0.01$) (Figure 3B). The experiment was repeated three times with similar outcomes (not shown).

3.3 Antibiotic selection enriches populations of transgenic schistosomules

Given that VSVG-MLV transduced schistosomules were rescued in the presence of G418, we investigated whether this rescue was reflected by enrichment of transgenic schistosomes in populations cultured in the antibiotic (Semple et al., 2010). To investigate this, we analyzed the transgene copy numbers in virion-transduced schistosomules cultured in increasing concentrations of G418. Genomic DNAs (gDNAs) were extracted from worms cultured in G418 for six and 10 days (from the experiment in Figure 3A). *NeoR* transgene copy numbers per nanogram of gDNA estimated by qPCR from these schistosomes are presented in Figure 3C. By day 6, there was an increased number of *neoR* transgenes in chromosomes of the parasites and the copy number increased directly in relation to increasing concentration of G418. Thus, a *neoR* copy number of 59 copies/ ng of gDNA was detected in gDNA from MLV transduced schistosomules cultured without G418, whereas at concentration of 125, 250, 500 and 1000 µg/ml G418, copy numbers for neoR of 73, 108, 149 and 105, respectively, were detected. A similar trend where copy number increased as G418 concentration increased was also evident in schistosomules cultured for 10 days (not shown).

3.4 Transgene expression in virion-transduced parasites reflects enrichment at the RNA level

End-point reverse transcription PCR and quantitative reverse transcription PCR (qRT-PCR) were used to investigate transcription activity of the neomycin resistance gene in the VSVG-MLV transduced schistosomules. Parasites not transduced with MLV virions (control), and schistosomules transduced with two increasing concentrations of virions (8×10^5 cfu [=1X] and 2.4×10^6 cfu [=3X] were cultured in 500 µg/ml of G418 for 10 days. Analysis by ethidium stained gels of the end-point PCR products revealed a signal at 383 bp in RNA from schistosomules transduced with the MLV virions, and indeed the signal strength increased in relation to the viral titer employed to transduce the schistosomules: worms transduced with the 3X titer showed much stronger *neoR* transcript signals than schistosomules transduced with 1X titer virions (Figure 4A). Signals at 224 bp representing the control target actin transcript were present in each of the three groups, indicating the integrity of RNA preparations (Figure 4A). No bands were detected in control groups where RNA was used as template, indicating absence of contaminating gDNAs in templates (not shown).

Expression of *neoR* relative to the endogenous reference gene *Sm*GAPDH from VSVG-MLV transduced schistosomules was investigated by qRT-PCR. In similar fashion to the outcome of the end-point PCR, real time PCR analysis detected higher levels of *neoR* transcripts in RNA from schistosomules transduced with higher compared to lower titers of virions (Figure 4B). In addition, *neoR* expression was elevated in schistosomules cultured in G418 compared to the virion-transduced worms cultured without G418, a trend seen with both high and low titers of virions (Figure 3B, dark blue versus light blue bars). This finding may reflect, at the RNA level, the enrichment of *neoR* transgenes and selection of transgenic schistosomes by the antibiotic.

Indeed, in this experiment, significantly more virion treated than non-virion schistosomes survived at day 10 (not shown).

4. Discussion

The availability of drug selection of transgenic schistosomes would be welcomed because it would provide a means to enrich for the presence of transgenic worms in transgene-exposed populations of parasites. Given that the retroviral transgene constructs employed here carried the *neoR* gene, we investigated the sensitivity of schistosomes to geneticin (G418), resistance to which is encoded by the *neoR* gene. G418 was toxic for schistosomules at each concentration tested from 125 – 1,000 µg/ml, a toxicity profile comparable to *C. elegans* and several other free-living nematodes and mammalian cells (Giordano-Santini et al., 2010; Yallop and Svendsen, 2001). G418 is derived from *Micromonospora rhodorangea*; it is an aminoglycoside antibiotic like neomycin and kanamycin, all of which are widely used for selection of resistant cells. In addition to the bactericidal effect, the aminoglycosides affect mitochondrial ribosomes of mammalian cells, blocking polypeptide synthesis leading to protein misreading (Smith and Baker, 2002; Padilla and Burgos, 2010).

Whereas other methods have been reported for determination of drug sensitivity of cultured schistosomes, including differential uptake of fluorophores (Peak et al., 2010), movement based (Smout et al., 2010) and calorimetry based (Manneck et al., 2011) assays, direct observation and counting of up to 200 schistosomules per treatment allowed us to establish toxicity of G418 and rescue of transgenic worms. This method allows the observer to appreciate the detail and anatomy of the schistosomules. This is tractable when the numbers to be analyzed are not overwhelming, when very high throughput might not be needed, and when the system has

to be validated. Indeed, given that this appears to be the first report of analysis of sensitivity of schistosomes to G418, direct observation was a judicious approach. (We observed little or no differences in outcomes when scoring schistosomules as dead or live by direct observation of unstained cultures, staining with Trypan blue [Figure S1] and/or differential staining with propidium iodide and fluorescein diacetate (Figure 1 and not shown.)

We tested if transgene *neoR* could rescue schistosomules cultured in G418. There was a clear trend - with each concentration of G418 examined, the retroviral transgene rescued the virion exposed worms from the antibiotic. For example, at day 4 in 1000 µg/ml G418, 80% of the MLV transduced parasites survived compared to 58% of the controls ($p \leq 0.05$); and at day 8 in 500 µg/ml G418, 90% of the retrovirus-transduced parasites remained alive compared to 62% of the control parasites (62%) ($p \leq 0.01$). Transgene copy number and transgene expression may reflect enrichment effect at DNA and RNA level respectively. In addition to the selection of antibiotic resistant schistosomes following retroviral transduction, we examined genomic DNA from the worms exposed to G418 for 6 and 10 days. Increasingly higher copy numbers of transgenes were present at each drug concentration from 125 to 1,000 at both 6 and 10 days. The findings confirmed the enrichment of *neoR* copy number – increasing density of the transgene was detected per ng of genomic DNA as the concentration of G418 increased. Enrichment of *neoR* copy number is clearly anticipated because non-transgenic flukes will be progressively eliminated from the cultured population. A similar enrichment of antibiotic resistance genes was reported in transgenic *C. elegans* cultured on puromycin (Semple et al., 2010). In addition, transcription of *neoR* increased in virion exposed schistosomules cultured on G418 compared to virion exposed schistosomules cultured without the antibiotic. This likely reveals enrichment of transgenic schistosomules within the population of transduced parasites subjected to G418

pressure (Yu et al., 1996). It likely reflects enrichment at the DNA level, not a conditional response to the antibiotic (Yallop and Svendsen, 2001).

Inclusion in culture media of Triton X-100 at 0.1% strikingly improved the selection of drug resistant *C. elegans*; the detergent facilitated access of the puromycin through the nematode cuticle (Semple et al., 2010). However, this detergent based advance with *C. elegans* remained unavailable for schistosomes since Triton X-100 rapidly killed schistosomules. Given that the tegument of the schistosomes includes a double membrane covering a syncytial, metabolically active cytoplasm, and of crucial importance for modulation of the host response and parasite survival (Gobert et al., 2003; Skelly and Wilson, 2006; Van Hellemond et al., 2006), it may not be surprising that exposure of schistosomules even to dilute concentrations of this detergent rapidly killed the parasites.

Antibiotic-resistance genes are commonly used as markers to monitor the introduction of exogenous genetic material into cells. Though widely used for genetic manipulation of cultured eukaryotic cells (Yallop and Svendsen, 2001), yeast and bacteria (Agaphonov et al., 2010), antibiotic selection systems have not so far been used for helminth parasites and were only recently reported with *C. elegans* and related free living nematodes (Giordano-Santini and Dupuy, 2011). With *C. elegans*, most genetic markers for transgenesis are based on readily scored visual phenotypes. In general such markers fail to provide a selective advantage, making the enrichment and maintenance of transgenic populations time-consuming. For antibiotic selection in *C. elegans*, Semple et al. (2010) predicted that, as with single-celled microbes, transgene endowed puromycin resistance will have wide-ranging potential for screening applications. Selection is also possible with resistance markers for other antibiotics (Semple et

al., 2010). As such, drug selection should be broadly applicable and constructive to the community of platyhelminth researchers.

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Figure legends

Figure 1. Evaluation of vitality of schistosomules of *Schistosoma mansoni* cultured in vitro and/or in the presence of G418. Panels A-D. Schistosomules cultured in the presence of G418. Representative micrographs of live (panel A, green arrow; panel B) and dead (A, red arrow; panel C) schistosomules. Images of representative Trypan blue stained schistosomules, panel D. Panels E-H. Representative schistosomules in culture for eight days and co-stained with propidium iodide (PI, 2.0 µg/ml) and fluorescein diacetate (FDA, 0.5 µg/ml). Epi-fluorescence and bright field microscopy was used to monitor uptake of fluorophores and examine schistosomules morphology. Panel E: Bright field. Panel F: Live schistosomules detected using a 494 nm filter. Panel G: Dead schistosomula detected with a 536 nm filter. Panel H: Merge of images in panels E-G. Red arrows indicate representative dead schistosomules and green arrows indicate representative live schistosomules. Scale bars, 50 µm (A-C), 100 µm (D), 200 µm (E-H).

Figure 2. G418/ geneticin dose response curve for schistosomules of *Schistosoma mansoni*. Panel A. Schematic showing the experimental design. Micrographs were captured on days highlighted with the blue boxes. ct: cercarial transformation to produce schistosomules. Panel B. Survival curve showing percentage of live schistosomules plotted against time in culture. Concentrations of G418 are indicated. Bars on curves represent one standard deviation (SD) of the mean. ANOVA and Student's *t*-test were performed among groups cultured in the presence of the indicated concentration of G418 and controls without the antibiotic. * $p \leq 0.05$, ** $p \leq 0.01$ Panel C: Representative images of schistosomules cultured in the presence of G418 for 8

480 days; 1: 0 µg/ml, 2: 125 µg/ml, 3: 250 µg/ml, 4: 500 µg/ml, 5: 1000 µg/ml G410. Scale bars, 200
481 µm.
482
483
484 **Figure 3. Survival of vesicular stomatitis virus glycoprotein (VSVG) pseudotyped murine**
485 **leukemia virus (MLV) transduced schistosomules in the presence of G418/ geneticin.** Panel
486 A. Percentage of live, VSVG MLV-transduced schistosomules and control non-virion exposed
487 schistosomules cultured for 10 days in increasing concentrations of G418. Concentrations of
488 G418 are indicated with discrete colors. ANOVA and Student's *t*-test were performed among
489 retrovirus transduced and control schistosomules cultured in the indicated concentration of G418.
490 * $p \leq 0.05$, ** $p \leq 0.01$ control: control schistosomules, MLV: VSVG-MLV transduced
491 schistosomules. Panel B: representative images of MLV transduced schistosomules (MLV 500)
492 and control schistosomules (control 500) cultured for 8 days in the presence of 500 µg/ml G418.
493 Scale bar, 200 µm. Panel C: neomycin resistance gene (*neoR*) copy number per nanogram of
494 genomic DNA from VSVG-MLV transduced schistosomules cultured for six days in increasing
495 concentrations of G418.
496
497
498 **Figure 4. Expression of neomycin transgene in retrovirus virion transduced schistosomules.**
499 Panel A: end point RT-PCR showing reporter gene expression from schistosomules not
500 transduced with virions (control), or 1X and 3X concentrations of VSVG-MLV virions (8×10^5
501 cfu [=1X] and 2.4×10^6 cfu [=3X]). Molecular size standards are shown at the left, while
502 amplicons of the expected sizes for the neomycin resistance gene (neo) and the reference gene

actin were apparent. Panel B: neomycin resistance gene expression relative to the endogenous *Sm* GAPDH observed in indicated group of VSVG-MLV transduced parasites. Bars, ± 1 standard deviation (SD).

Supplementary materials

Figure S1. Trypan blue staining of schistosomules. Representative micrographs of Trypan blue stained (A, B) or non-stained schistosomules (C, D) cultured for 10 days without G418 (A, C) or in 500 µg/ml G418 (B, D). Green arrows indicate live and red arrows indicate dead schistosomules. Scale bars, 200 µm.

Figure S2. G418 dose response curve for schistosomules. Panels A to E: representative micrographs of schistosomules cultured in the presence of the G418 (various concentrations) but without Triton X-100. Panel F: images of schistosomules cultured in 0.5% Triton X-100 without G418. Scale bars, 200 µm.

Figure 1

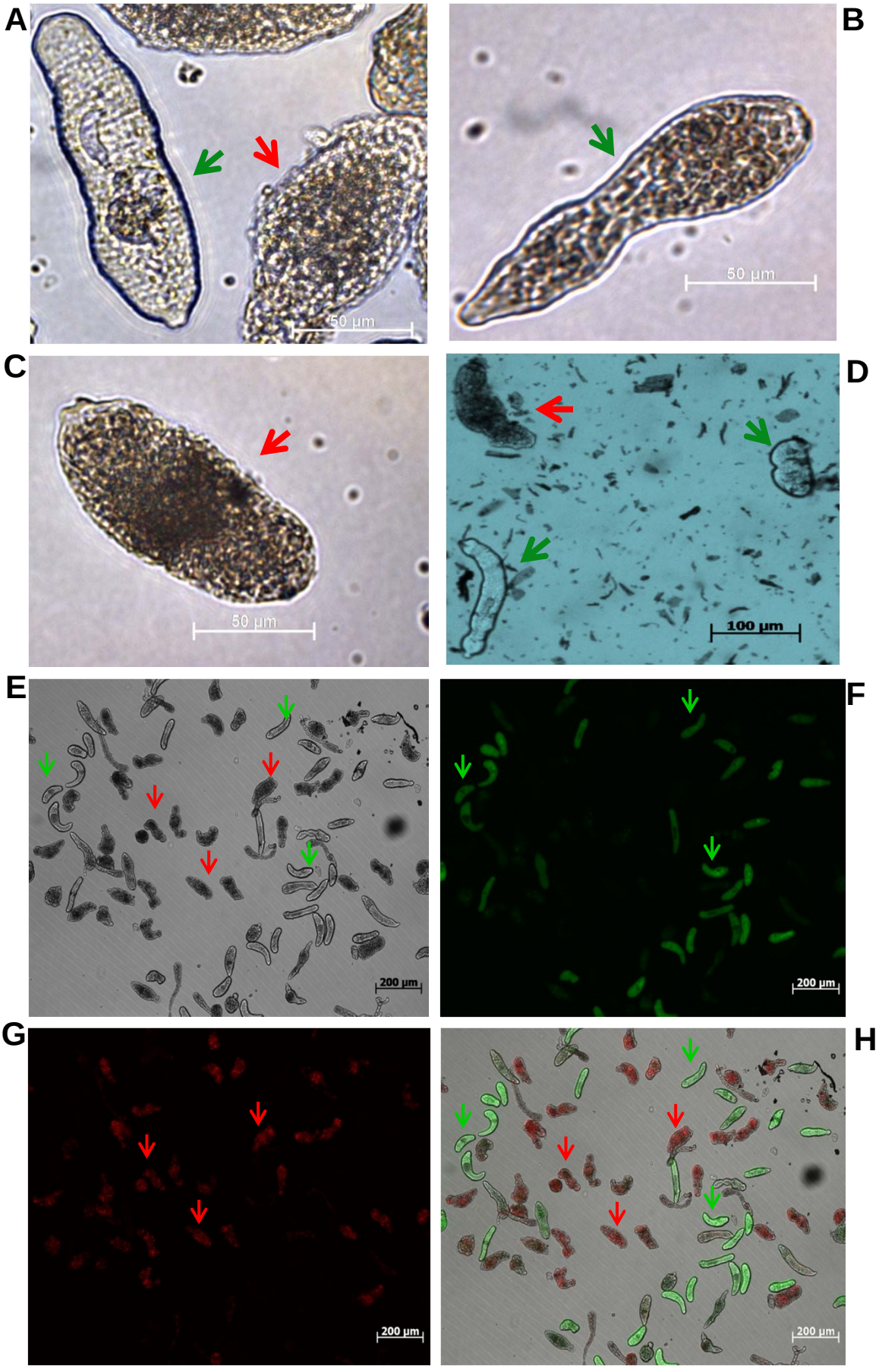
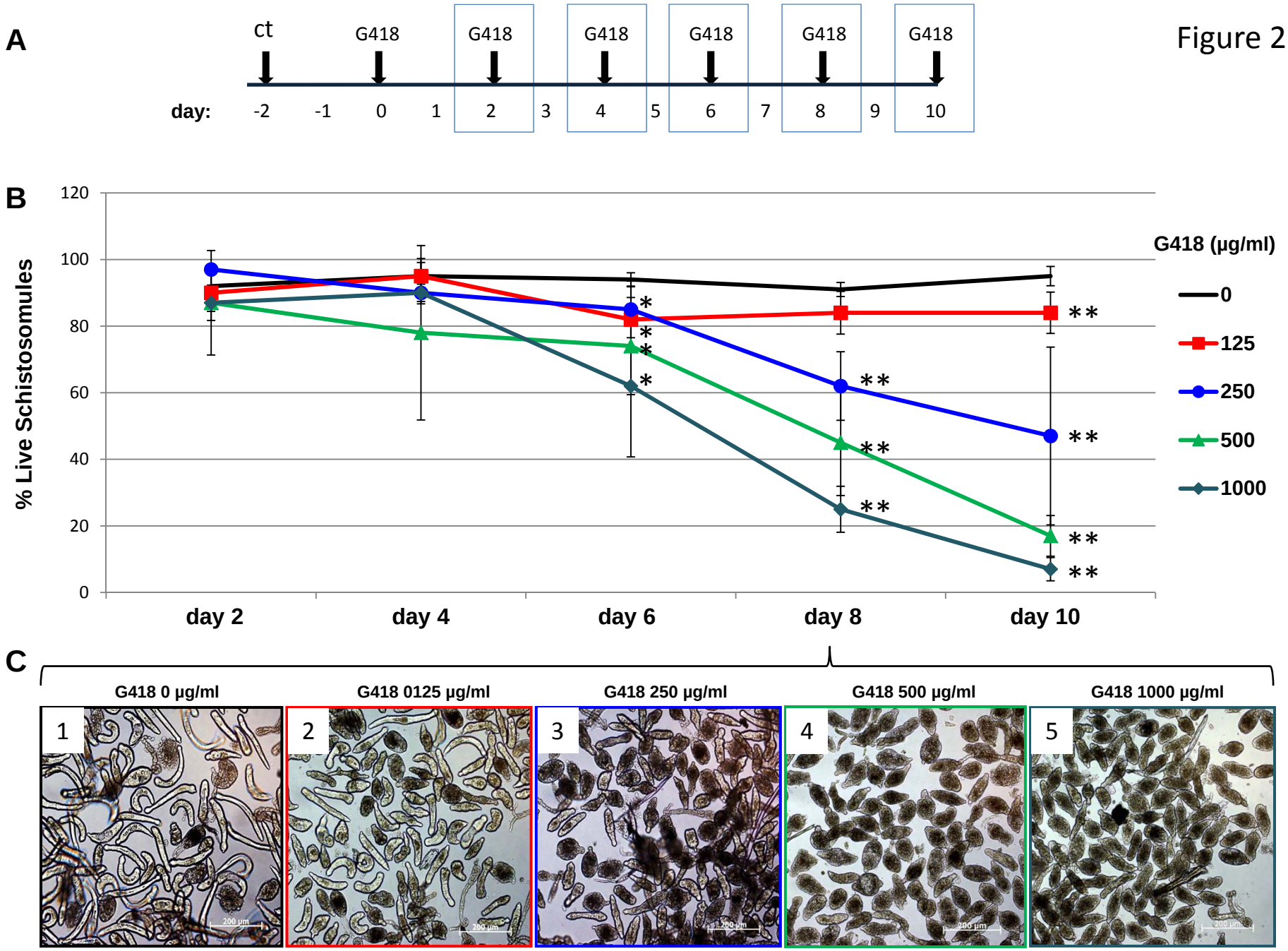


Figure 2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G418
G418
G418
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G418
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-1
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% Live Schistosomules
120
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40
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day 2
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day 10
G418 (µg/ml)
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1000
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G418 1000 µg/ml
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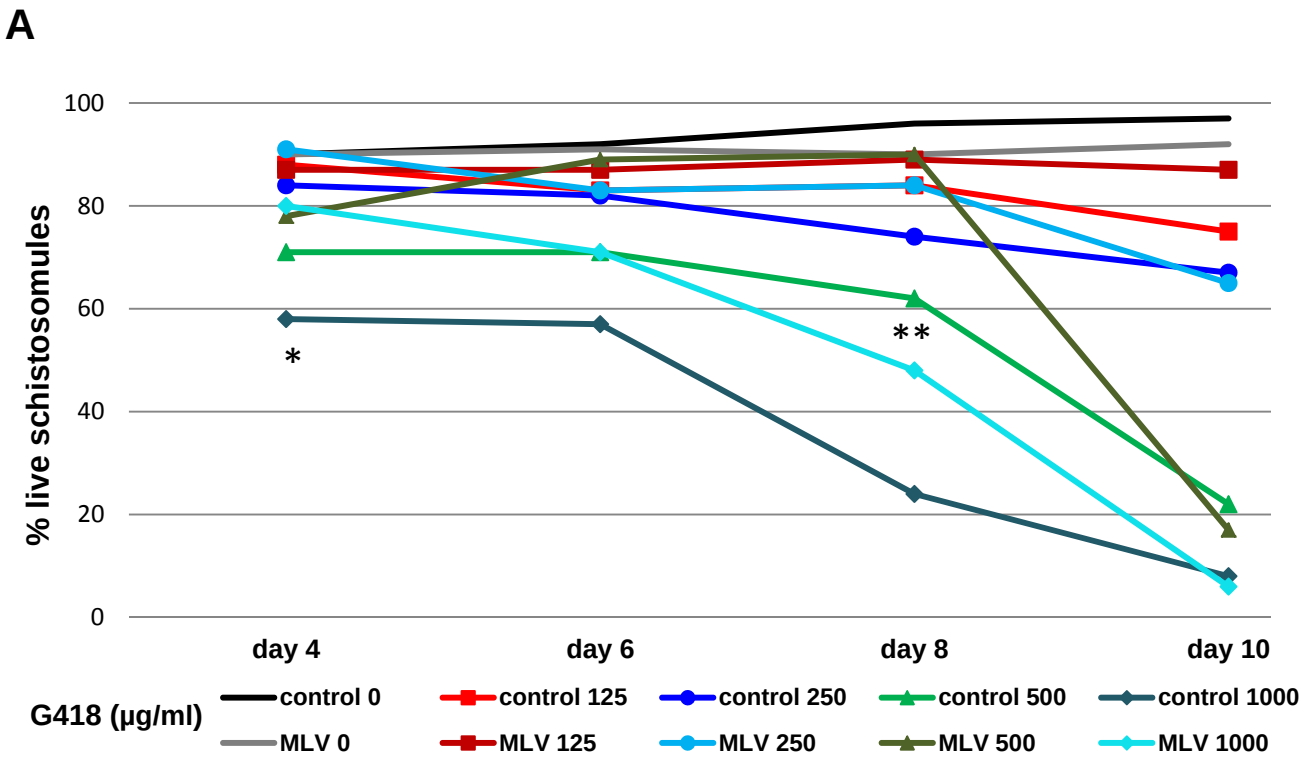
5

Figure 3


A
100
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day 4
day 6
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G418 (µg/ml)
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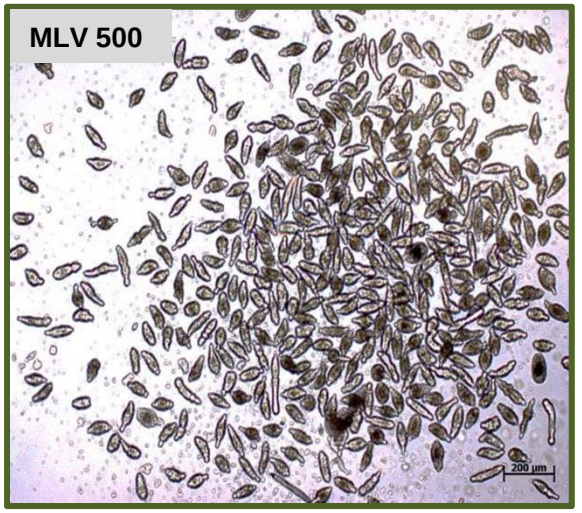
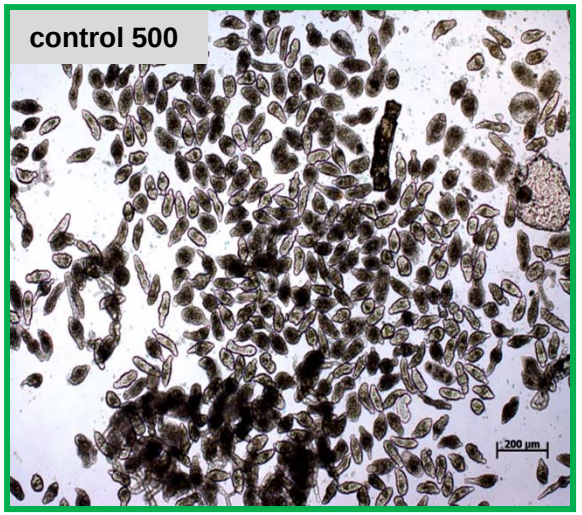
control 500
control 1000
MLV 0
MLV 125
MLV 250
MLV 500
MLV 1000


B
day 8
MLV 500
200 µm


control 500
200 µm

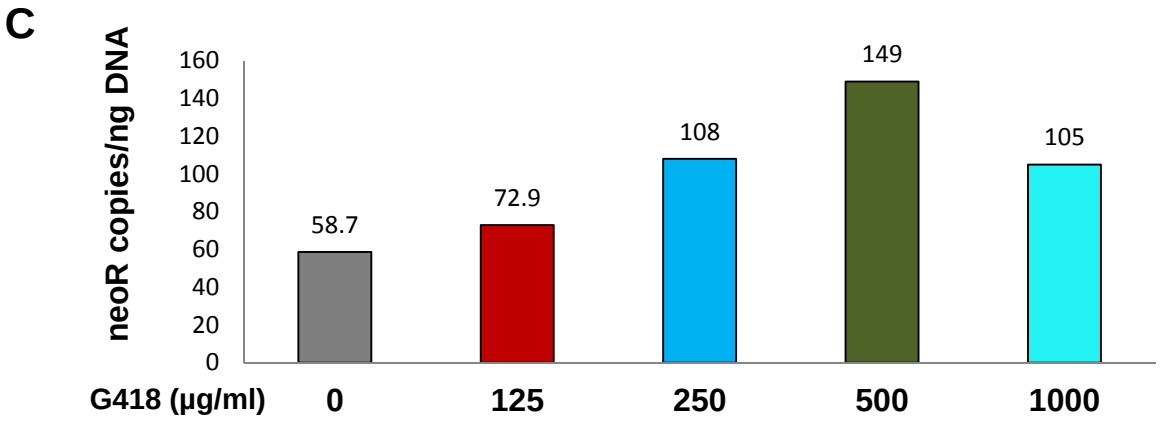
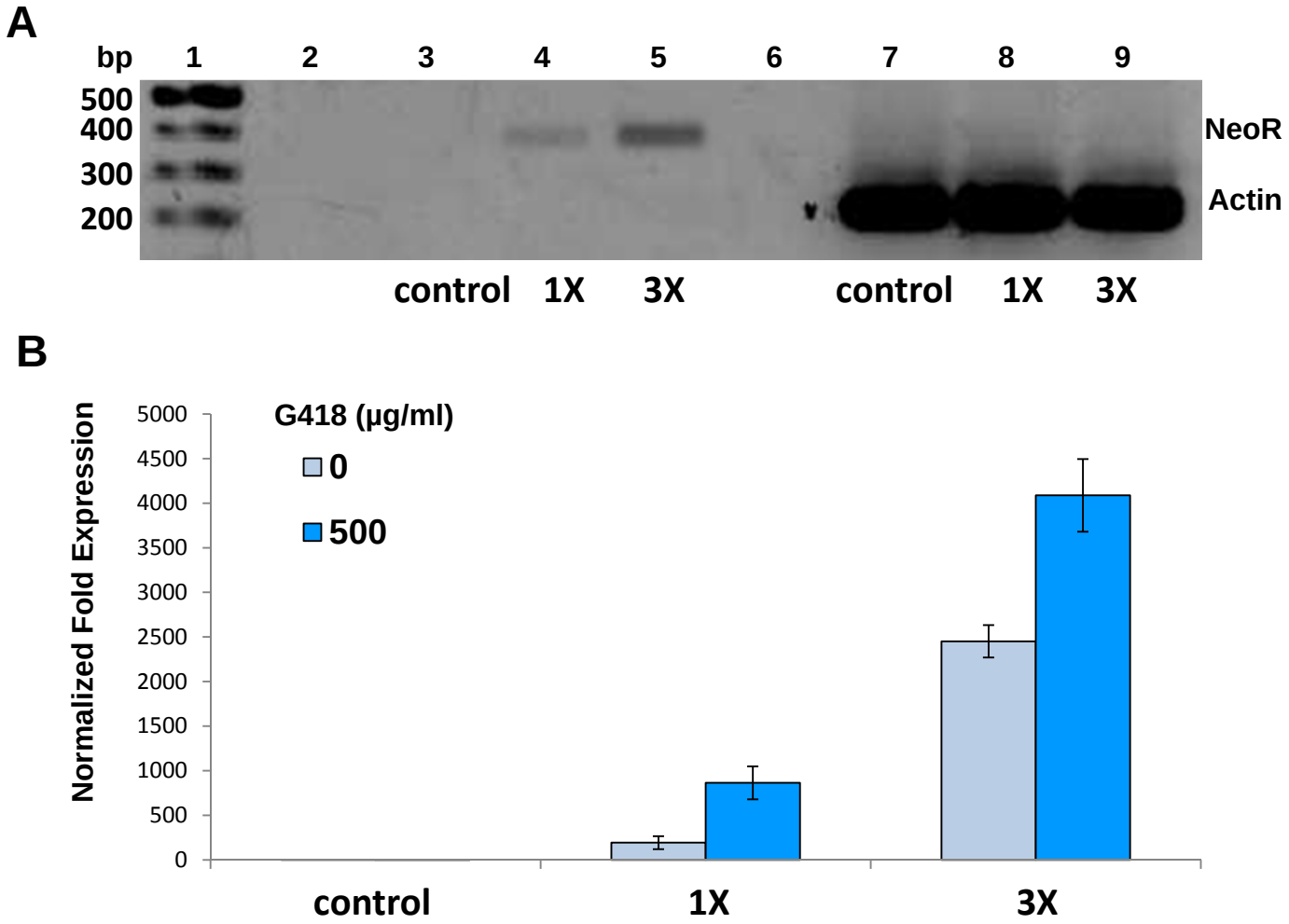

C
neoR copies/ng DNA
160
140
120
100
80
60
40
20
0
58.7
72.9
108
149
105
G418 (µg/ml)
0
125
250
500
1000

Figure 4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NeoR
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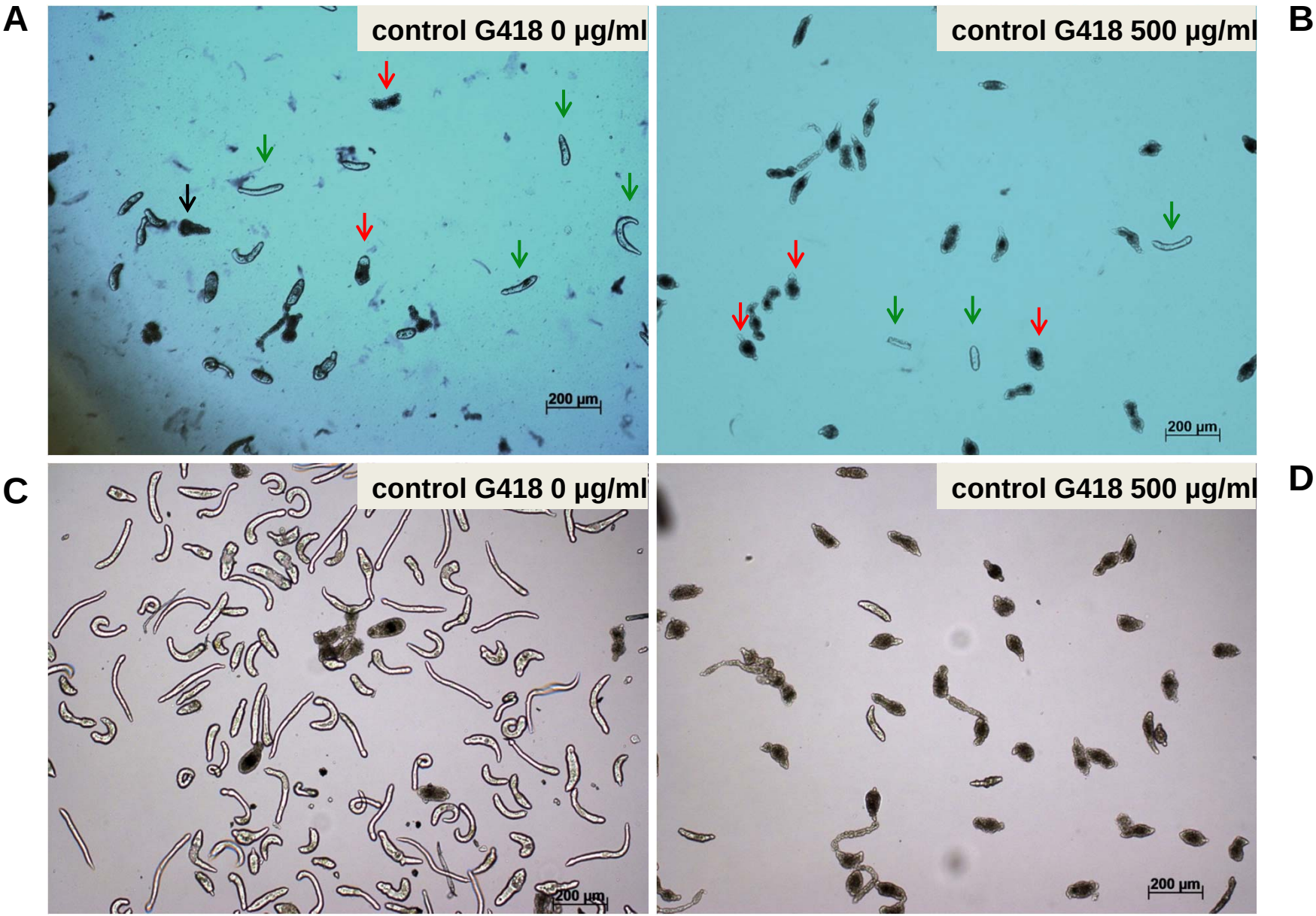
1X
3X
B
Normalized Fold Expression
G418 (µg/ml)
0
500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
control
1X
3X

Figure S1. Trypan blue staining of schistosomules of *Schistosoma mansoni*. Representative micrographs of Trypan blue stained (A, B) or non-stained schistosomules (C, D) cultured for 10 days without G418 (A, C) or in 500 µg/ml G418 (B, D). Green arrows indicate live and red arrows indicate dead schistosomules. Scale bars, 200 µm.



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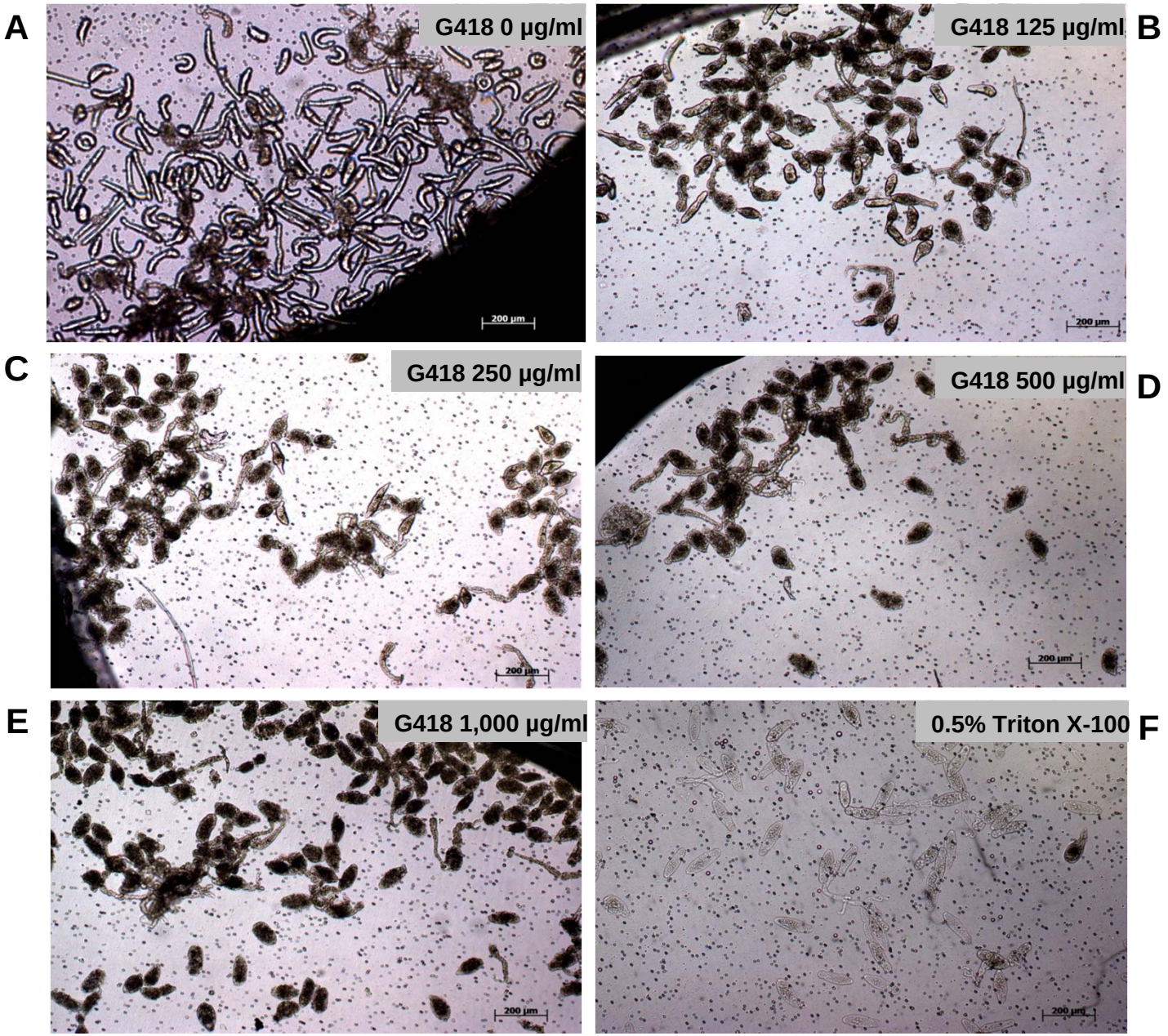

A.4. Análisis de la expresión de genes reporteros en parásitos transgénicos

ARTICULO: El aislante de cromatina cHS4 protege a los transgenes retrovirales del silenciamiento en *Schistosoma mansoni*

Sutas Suttiprapa*, **Gabriel Rinaldi*,** Paul J. Brindley

(* Igual contribución al trabajo)

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ORIGINAL PAPER

Prototypic chromatin insulator cHS4 protects retroviral transgene from silencing in *Schistosoma mansoni*

Sutas Suttiprapa · Gabriel Rinaldi · Paul J. Brindley

Received: 1 July 2011 / Accepted: 31 August 2011

Abstract Vesicular stomatitis virus glycoprotein (VSVG) pseudotyped murine leukemia virus (MLV) virions can transduce schistosomes, leading to chromosomal integration of reporter transgenes. To develop VSVG-MLV for functional genomics in schistosomes, the influence of the chicken β-globin cHS4 element, a prototypic chromatin insulator, on transgene expression was examined. Plasmid pLNHX encoding the MLV 5′- and 3′-Long Terminal Repeats flanking the neomycin phosphotransferase gene (*neo*) was modified to include, within the U3 region of the 3′-LTR, active components of cHS4 insulator, the 250 bp core fused to the 400 bp 3′-region. Cultured larvae of *Schistosoma mansoni* were transduced with virions from producer cells transfected with control or cHS4-bearing plasmids. Schistosomules transduced with cHS4 virions expressed 2–20 times higher levels of *neo* than controls, while carrying comparable numbers of integrated proviral transgenes. The findings not only demonstrated that cHS4 was active in schistosomes but also they represent the first report of activity of cHS4 in any Lophotrochozoan species, which has significant implications for evolutionary conservation of heterochromatin regulation. The findings advance prospects for transgenesis in functional genomics of the schistosome genome to discover intervention targets because they provide the means to enhance and extend transgene activity including for vector based RNA interference.

Keywords Schistosome · Pseudotyped retrovirus · cHS4 insulator · Transgene

Sutas Suttiprapa and Gabriel Rinaldi contributed equally to this article.

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Introduction

Draft genome sequences have become available for two of the major species responsible for schistosomiasis, *Schistosoma japonicum* and *Schistosoma mansoni* (Berriman et al. 2009; *S. japonicum* Genome and Functional Analysis Consortium 2009). The schistosome genomes are 363–397 MB in size, and include ~13,000 protein encoding genes, of which the functions of only a small percentage have been

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characterized in depth (Han et al. 2009). Unlike for prokaryotic and unicellular eukaryotic pathogens, functional genomics toolkits are not well advanced for schistosomes, or indeed for the helminth parasites at large. We have shown that vesicular stomatitis virus glycoprotein pseudotyped murine leukemia virus (VSVG-MLV) virions can transduce different developmental stages of *S. mansoni* including adult worms, schistosomules and eggs, leading to integration of reporter proviral transgenes into schistosome chromosomes (Kines et al. 2008; Kines et al. 2010; Rinaldi et al. 2011). Moreover, VSVG-MLV has recently shown utility in vector based RNA interference in adult schistosomes (Tchoubrieva et al. 2010) and for expression of oncogenes in larval blood flukes (Yang et al. 2010). Indeed, although other approaches are also in development for functional genomics of parasitic helminths (Dvorak et al. 2010; Lok 2009; Morales et al. 2007), pseudotyped MLV appears more well advanced at this time. The forward genetics strategy of insertional mutagenesis using integration proficient vectors such as VSVG-MLV represents a powerful approach to functional genomics (see Ivics et al. 2009).

There appear to be clear advantages to deployment of gammaretrovirus virions for functional genomics, including the infectious nature of retroviruses which facilitates transduction of the target organism, broad host and tissue ranges endowed by VSVG, and potential to produce virions at high titer (Amsterdam and Hopkins 2006; Mann et al. 2011; Mattison et al. 2010). However, there also are potential limitations; gammaretroviruses cannot transduce non-dividing cells well, transgene activity is influenced by positional effects within chromosomes, and attenuation of transgene expression can occur over time (e.g., Nabekura et al. 2006; Persons 2009). To advance the efficacy of VSVG-MLV for functional genomics of *S. mansoni*, we investigated activity of the cHS4 insulator (Chung et al. 1993) as a component of the proviral transgene for transduction of cultured schistosomules. cHS4, a nuclease hypersensitive region from the globin gene locus of the chicken is known as a barrier element that can protect against transgene silencing in mammalian and insect cells (Arumugam et al. 2009; Chung et al. 1993). A core objective of transgenesis as functional genomic tool is to achieve robust, sustained expression of the integrated transgenes. Chromatin insulators can suppress or inhibit transgene silencing not only for reporter genes such as *neo* or luciferase but also shRNA encoding cassettes for vector based RNA interference. We describe a new MLV construct, which includes a functional yet minimally sized, cHS4 element inserted within the retroviral 3′-LTR and report that, after transduction of schistosomules, transgene expression of the neomycin phosphotransferase gene (*neo*) was protected from silencing.

Materials and methods

Parasites

Biomphalaria glabrata snails infected with the NMRI (Puerto Rican) strain of *S. mansoni* were supplied by Dr. Fred Lewis, Biomedical Research Institute, Rockville, MD. Schistosomules were obtained from cercariae released from the snails, and cultured as described (Mann et al. 2010). In brief, cercariae were concentrated by centrifugation (800 g/10 min) and washed with schistosomule wash medium (Dulbecco's modified Eagle's medium supplemented with 200 units/ml of penicillin, 200 µg/ml of streptomycin, 500 ng/ml of amphotericin B and 10 mM HEPES). Cercarial tails were sheared off by 20 passes back and forth through a 22G emulsifying needle, after which schistosomule bodies were separated from tails by Percoll gradient centrifugation (Lazdins et al. 1982). Schistosomula were washed three times in wash medium and cultured at 37°C under 5% CO_2 in Basch's medium (Basch 1981) supplemented with washed human erythrocytes, one µl packed red cells per ml culture medium. Control and virion exposed schistosomules were examined, and images recorded, under bright light using a Zeiss Axio Observer A.1 inverted microscope fitted with a digital camera (AxioCam ICc3, Zeiss).

Plasmid constructs for cHS4-insulated murine leukemia virus gammaretrovirus

A 650 bp portion of full-length cHS4, comprising the 250 bp of core DNA sequence from the 5′ end of cHS4 fused with 400 bp from the 3′ end of cHS4 was chemically synthesized (GENEART AG, Regensburg, Germany). Plasmid pLNHX (Clontech, Mountain View, CA) was digested with *Xho*I to remove the Drosophila heat shock 70 gene promoter, to produce

pLNHXΔD70. The 650 bp portion of cHS4 was inserted into the *Xba*I site of the U3 region of the 3′-LTR region of pLNHXΔD70 to derive pLNHX_cHS4_650.

Transduction of schistosomes with pseudotyped virions

Production of VSVG-pseudotyped virions in packaging cells was undertaken as described (Kines et al. 2006). In brief, GP2–293 (modified HEK-293) packaging cells (Clontech) were transfected with pLNHX_ΔD70 or pLNHX_cHS4_650 along with a plasmid encoding VSVG, delivered in liposomes (Lipofectamine 2000, Invitrogen). Subsequently, virions in the culture supernatants were concentrated by high-speed centrifugation (Sorvall, SS-34 rotor) 50,000×*g*, 90 min, at 4°C. Pellets of concentrated virions were resuspended in modified Basch's medium at 4°C overnight, aliquoted and stored at −80°C. Viral titers were determined by real time PCR targeting viral genome and/or by functional assay using NIH-3T3 fibroblast cells cultured in the presence of G418/geneticin (Mann et al. 2011; Rinaldi et al. 2011).

Schistosomula ($2 - 5 \times 10^4$), at 1–7 days after transformation from cercariae, were cultured in 6 well plates containing one ml of virion preparation with an infectivity of 1×10^6–1×10^7 colony forming units (CFU)/ml, using similar CFUs of parental (from pLNHX_ΔD70) or cHS4 bearing (pLNHX_cHS4_650) virions in the presence of the cationic polymer polybrene. A control group of schistosomules treated with polybrene B in the absence of virions was included. After exposure to virions for 18 h, culture media were replaced with virion-free medium supplemented with washed human erythrocytes. Thereafter, culture media were replaced every second day. Retrovirus-exposed schistosomules were harvested at various intervals after transduction, snap frozen and then stored at −80°C.

Transgene copy number

Genomic DNA (gDNA) was extracted from virion transduced and untreated, control schistosomules using a kit (E.Z.N.A. SQ Tissue DNA Kit, Omega Bio-tek), and gDNA concentrations determined by spectrophotometer (NanoDrop 1000). Quantitative PCRs were performed using TaqMan primers and probes specific for *neo* (forward primer, GGA GAG GCT ATT CGG CTA TGA C; reverse primer. CGG ACA GGT CGG TCT TGA C; probe, 5′-/56-FAM/CTG CTC TGA TGC CGC CGT GTT CCG/3IABIk_FQ/-3′) and *S. mansoni* glyceraldehyde 3-phosphate dehydrogenase (SmGAPDH; GenBank M92359) (forward primer, TGT GAA AGA GAT CCA GCA AAC; reverse primer, GAT ATT ACC TGA GCT TTA TCA ATG G; probe, 5′-/56-FAM/AAG ACT CCA GTA GAC TCA ACG ACA T/3IABIk_FQ/-3′). Reactions were carried out using 100 ng of template gDNA in 20 µl volumes along with Perfecta qPCR FastMix, UNG (Quanta Bioscience) and primer-probe sets. Quantitative PCRs were performed in triplicate, using 96-well plates (Bio-Rad), with a denaturation step at 95°C of 3 min followed by 40 cycles of 30 s at 95°C and 30 s at 55°C, in thermal cycler (iCycler, Bio-Rad) fitted with a real time detector (iQ5, Bio-Rad). The relative quantification assay $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), with SmGAPDH as the reference gene, was used to ascertain the relative transgene copy number of *neo* in gDNAs of schistosomules transduced with parental (from pLNHX_ΔD70) or cHS4 chromatin insulator bearing (pLNHX_cHS4_650) virions. Relative *neo* copy number in viral treated groups reflect the fold change of *neo* copy number normalized to the *Sm*GAPDH reference gene and relative to the untreated control group (calibrator).

Expression of retroviral transgene

Total RNAs were extracted from pellets of frozen schistosomula using a kit (RNAqueous-4PCR, Ambion) and their concentrations estimated by spectrophotometer (NanoDrop 1000). cDNAs were synthesized from 500 ng RNA using the iScript cDNA Synthesis Kit (Bio-Rad). End point PCR targeting the *neo* gene encoding neomycin phosphotransferase (forward primer, TGT GCT CGA CGT TGT CAC TGA A; reverse primer, ATG AAT CCA GAA AAG CGG CCA) was performed using the Promega GoTaq Green Master mix system, with 35 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 90 s and final extension at 72°C for 7 min. The *S. mansoni* actin 2 gene, GenBank U19945 (forward primer, CAG TGT TCC CTT CCA TCG TT, reverse primer, GGA CAG GGT GTT CTT CTG GA) was used as a endogenous schistosome gene control for integrity of the cDNA. Two negative control templates were included in the assays: first, RNA that

was not reverse transcribed, and second, water was substituted for template cDNA. End-point PCR products were sized by electrophoresis in 1% agarose, after which images of the ethidium bromide-stained gel were obtained (Gel-Doc XR system, Bio-Rad). Quantitative PCRs for expression of *neo* and GAPDH were performed as above for determination of transgene copy number except that cDNA reverse transcribed from 500 ng of total RNA was employed as the PCR template. The relative quantification assay $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) using SmGAPDH as the reference gene was employed to estimate the relative expression of *neo* in schistosomules transduced with parental (from pLNHX_ΔD70) or cHS4 chromatin insulator bearing (pLNHX_cHS4_650) virions. Relative *neo* expressions in viral treated groups reflect the fold change of *neo* expression normalized to the *Sm*GAPDH reference gene and relative to the untreated control group (calibrator).

Bioinformatics

The amino acid sequences of human CCCTC-binding factor (CTCF; GenBank AAB07788), upstream transcription factor 1 (USF1; CAI15372) and vascular endothelial zinc finger 1 (VEZF1; NP_009077) which are known to interact with cHS4 and other chromatin insulators in other species [e.g., (Dickson et al. 2010; Heger et al. 2009; West et al. 2004)] were used as queries in Blastp searches http://www.blast.ncbi.nlm.nih.gov/Blast.cgi of the public databases, including the draft genome version 3.1 of *S. mansoni*, aiming to identify schistosome orthologues that might be capable of interacting with cHS4.

Results

Prototypic chromatin insulator cHS4 in U3 region of LTRs of MLV flanking *neo* transgene

Previously we demonstrated that pseudotyped MLV can transduce schistosomes, leading to integration of proviral transgenes in schistosome chromosomes (Kines et al. 2008). Earlier studies also had indicated that, in this situation, the 5′-LTR drove transcription of *neo* from the integrated provirus (Kines et al. 2006, 2008). It is notable that insertion of chromatin insulator sequences in the U3 region of the 3′-LTR of MLV results in two copies of the insulator in the proviral form of MLV (Rivella et al. 2000). However, the cHS4 element is 1.2 kb in length, sufficiently long to lead to reduced titers during production of the virions (Nielsen et al. 2009; Urbinati et al. 2009). Since shorter versions of cHS4 retain most or all of the insulator activity of the full length element (Aker et al. 2007; Arumugam et al. 2009), and have been included in retroviral and lentiviral vectors for mammalian cells (Aker et al. 2007; Arumugam et al. 2009), we employed a strategy that involved using part of the cHS4 element in a new construct for transduction of schistosomes. Our approach involved fusing the 250 bp core of cHS4 with the 3′-terminal 400 bp of the insulator, thereby halving the size of the insulator with the aim of retaining the ability to obtain high titers of virions (Aker et al. 2007; Arumugam et al. 2009; Li et al. 2009).

The retroviral vector pLNHX was modified to remove the D70 promoter (a Drosophila HSP70 gene promoter) resulting in a smaller plasmid termed, pLNHX_ΔD70 (insert size 5,301 bp). Thereafter, the shortened (650 bp) version of the 1.2 kb cHS4 insulator was inserted into the U3 region of the 3′-LTR of the retroviral insert of pLNHX, and this construct termed pLNHX_cHS4_650 (Fig. 1). The integrity of the modifications was verified by nucleotide sequence analysis; the insert of pLNHX_cHS4_650 has been assigned GenBank number JN000001. Virions encoded by the inserts of pLNHX_ΔD70 and pLNHX_cHS4_650 were produced and titers determined; in general comparable titers were produced from both plasmids, the non-insulator and the insulator bearing forms. Titers in the range of $1–10 \times 10^6$ CFUs can be reliably produced from both plasmids (not shown). The inserts of these plasmids have been trimmed so that they include only one promoter driving one reporter gene, in this case the 5′-LTR driving *neo* (Fig. 1). Schistosomula were exposed to these virions. As with mammalian target cells, we anticipated that, once reaching cytoplasm of schistosome cells, viral reverse transcriptase would have reverse transcribed the RNA genomes to proviral cDNAs and that during reverse transcription, the cHS4 insulator at U3 of 3′-LTR would have been copied and transferred to the 5′-LTR (Hantzopoulos et al. 1989; Rivella et al. 2000). We also anticipated that insulated DNA would integrate into schistosome chromosomes through the enzymatic activity of retroviral integrase (Fig. 1).

Equivalent transgene copy number in schistosomes transduced with insulated or control virions

Transgene copy number was investigated in schistosomes exposed to virions produced from the pLNHX_ΔD70 and pLNHX_cHS4_650 constructs. Schistosomules were collected three and 10 days after addition of virions. At this point, parasite populations were divided into two groups; gDNA was extracted from one, total RNA from the other. qPCR revealed the same or similar relative transgene copy number (i.e. *neo* copy number relative to the reference gene) in the gDNA from schistosomules exposed to either virion genotype: 23 and 24 copies in the day 3 groups, and 32 and 30 copies in the day 10 groups, of the pLNHX_ΔD70 and pLNHX_cHS4_650 virions, respectively (Fig. 2, panel a). Analysis by qPCR of RNAs from the correspondent samples revealed that the relative expression of *neo* in the cHS4 encoded virion transduced worms was approximately double that seen in the schistosomes transduced with the control virus. This

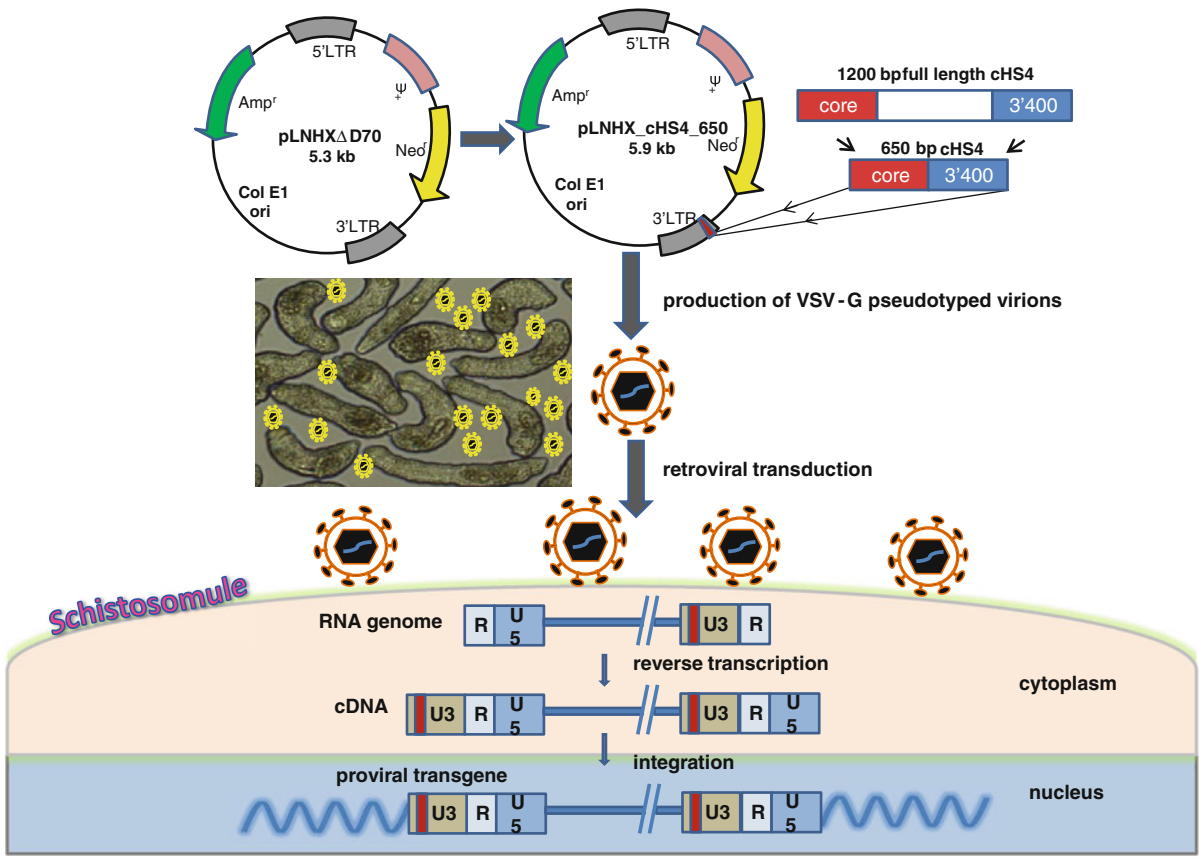

Fig. 1 Schematic overview of insulator vector and predicted outcome of virion transduction of schistosomes. The pLNHXΔD70 plasmid was constructed by removing Drosophila heat shock protein 70 promoter from pLNHX. The 650 bp active component of the chicken cHS4 insulator, 250 bp of core DNA sequence fused with 3′ 400 bp of cHS4 insulator was chemically synthesized as a linear DNA fragment, which was subsequently inserted into the U3 domain of the 3′-LTR of pLNHXΔD70 to derive plasmid pLNHXcHS4_650 (*top panel*). Replication incompetent VSVG-pseudotyped virions were produced in packaging cells. Schistosomules of *S. mansoni* were cultured with virions from pLNHXΔD70 to pLNHXcHS4_650 constructs (*center*). After transducing the schistosome surface (Kines et al. 2006), it is predicted that the gammaretroviral RNA genome is reverse transcribed to non-integrated proviral cDNA; and during this process, the 650 bp of cHS4 insulator is copied and transferred from 3′ to 5′-LTR to generate the viral construct containing insulator at both sides of LTR (Rivella et al. 2000). The proviral genome, carrying the *neo* transgene flanked by LTRs bearing the cHS4 chromatin insulator, approaches the nucleus at cell division, and integrates into a schistosome chromosome catalyzed by retroviral integrase

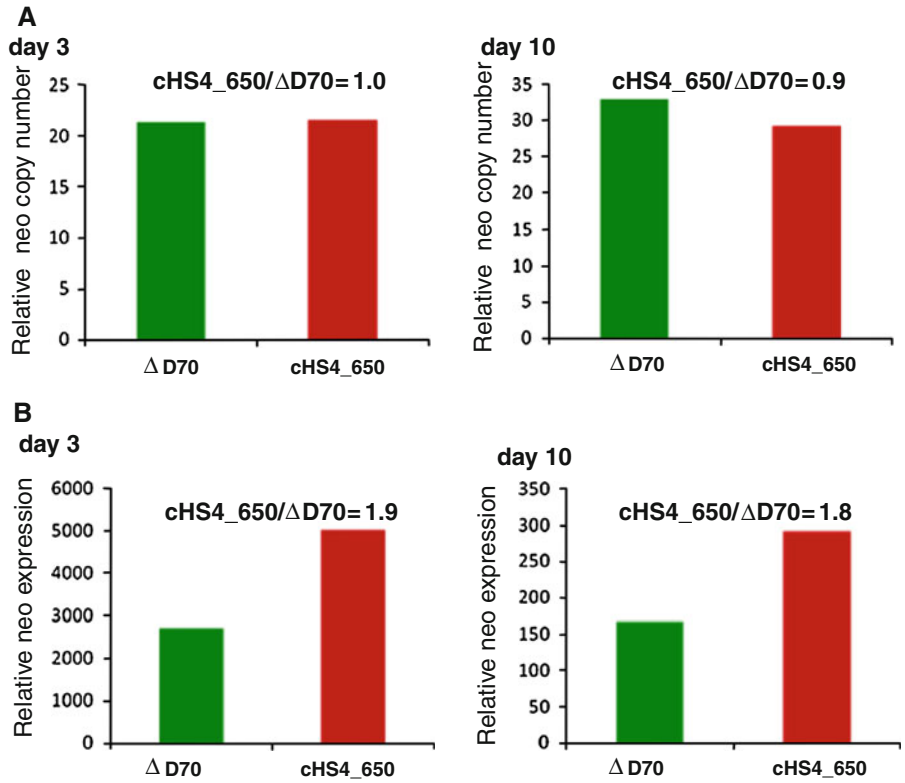

Fig. 2 Determination of relative transgene copy number and expression in schistosomules transduced with insulated and non-insulated gammaretrovirus virions by quantitative real time PCR. **a** Relative transgene copy number determined in genomic DNAs from schistosomules at 3–10 days after transduction by pseudotyped gammaretroviral virions; pLNHX_ΔD70 virions (*green bars*), pLNHX_cHS4_650 virions (*red bars*). **b** Expression of transgene *neo* detected in schistosomule groups shown in **a**. Ratios between the relative transgene copy numbers and transgene expression detected in schistosomules transfected with the insulated and non-insulated virions are shown in **a** and **b**, respectively. (Color figure online)

trend was evident at both three and 10 days after transduction with the pseudotyped virions (Fig. 2b). The experiment was repeated, and similar findings were obtained on both occasions (not shown). Interestingly, whereas higher expression of *neo* was seen at both time points, there was nevertheless a substantial reduction of the level of expression at day 10 compared to day 3 (Fig. 2b). Nonetheless, together these findings revealed that both virions, the parental version and the experimental form carrying the cHS4 chromatin insulator, were of comparable proficiency for transduction of schistosomules. We detected a similar density of proviral transgenes in the genomic DNA recovered from virion-exposed populations of schistosomules. More importantly, despite equivalent relative *neo* transgene copy number, they revealed substantially higher transgene expression from the cHS4-insulated proviral transgene than the control, non-insulated transgene.

Prototypic insulator cHS4 protects retroviral transgene from silencing in schistosomes

Next, we investigated the phenomenon of higher levels of expression of *neo* in schistosomules transduced with insulated proviral transgenes, over longer periods of time—at 5, 10 and 20 days after virus transduction. Total RNA extracted from schistosomula transduced with insulated and parental virions were reverse transcribed and used as template for PCR. Both endpoint RT-PCR and qRT-PCR were employed to visualize and quantify expression of the *neo* transgene. In analysis by endpoint PCR, ethidium-stained products showed stronger signals for *neo* transcripts in the cHS4 insulated virions compared to the control virions, at each interval after exposure. No differences were evident in expression of actin, a control target for RNA integrity. As expected, no *neo*

signal was observed in control schistosomules not exposed to virions (Fig. 3a). In like fashion, analysis by qRT-PCR confirmed higher expression of *neo* in schistosomes exposed to insulated compared to non-insulated virions; the ratio of *neo* expression in the pLNHX_cHS4_650 compared to pLNHX_ΔD70 virions was 3.2, 6.9 and 19.6 at 5, 10 and 20 days after transduction, respectively (Fig. 3b). Although, the relative expression of *neo* was substantially greater at each time point in schistosomes transduced with insulated compared with control virions, transgene expression globally diminished in all the transgenic parasites over time. Thus, cHS4 inhibited transgene silencing but did not completely block it. By light microscopy, no differences were apparent in the viability or appearance of schistosomules among the non-virus exposed control schistosomules, or the virion exposed groups (Fig. 3c); the representative micrographs in Fig. 3 show schistosomules at 10 days after exposure to the virions. The experiment was repeated, with similar gene expression being observed on both occasions (not shown).

Potential cHS4 binding proteins of schistosomes

Bioinformatic searches were carried for putative orthologues of the binding proteins CTCF, USF1 and VEZF1, revealing schistosome matches to each: CTCF, match accession numbers XP_002575256, XP_002578242, XP_002575460; USF1, accessions XP_002579915, XP_002578293, XP_002571520; and VEZF1, accessions XP_002577022, XP_002575460, XP_002575256 from *S. mansoni* with the *E*-values <1E − 4 and 28–44% similarities (Table S1).

Discussion

We have recently transduced the human blood fluke *S. mansoni* with a pseudotyped gammaretrovirus, murine leukemia virus (see Mann et al. 2011). Our aim is to use this approach for functional genomics of schistosomes, which are major neglected tropical disease pathogens of humanity and for which draft genome sequences have recently been reported (Berriman et al. 2009; Hotez et al. 2008; *S. japonicum* Genome and Functional Analysis Consortium 2009). The nature of the retroviral developmental cycle, in particular the integration of the proviral form into host chromosomes and target site preferences for insertion into host chromosomes are desirable attributes that make these vectors attractive for functional genomics and transgenesis (Roth et al. 2011; Wang et al. 2007). However, gammaretroviral and lentiviral vectors have drawbacks as reagents for functional genomic and gene therapy approaches, including transgene silencing due to position effects, oncogene activity, and silencing of the transgene cargo by proviral 5′-LTRs. Interest in retroviral vectors also is tempered, particularly in clinical settings, by issues of genotoxicity involving the activation of cellular oncogenes flanking sites of vector integration (Bohne and Cathomen 2008; Hacein-Bey-Abina et al. 2003; Li et al. 2009; Ott et al. 2006).

The cHS4 element, a well characterized vertebrate chromatin insulator from the control region of the chicken β-globin locus, contains a DNase-I hypersensitive site (cHS4) and constitutes the boundary of the b-globin locus (Chung et al. 1993; Reitman and Felsenfeld 1990). cHS4 exhibits classical insulator activities, including the ability to block the interactions of globin gene promoters and enhancers in cell lines, with the ability to protect expression cassettes in Drosophila, transformed cell lines, and transgenic mammals from position effects (Chung et al. 1993; Emery et al. 2000). The cHS4 insulator has been incorporated into retroviral and lentiviral gene therapy vectors aiming to improve performance and relieve other impediments (Desprat and Bouhassira 2009; Emery et al. 2002, 2000; Li and Emery 2008; Li et al. 2009). With retroviral vectors, constructs have been assembled by flanking the vector with the cHS4 element using a double-copy arrangement, wherein cHS4 elements were cloned into the 3′-LTR of the producer plasmid. From this arrangement, they are copied into the 5′-LTR during reverse transcription of the retroviral RNA genome and proviral integration, resulting in a proviral insertion where the 5′-LTR and internal transgene sequences are insulated from host genomic sequences on both sides of the proviral transgene and the 3′-LTR is insulated from genomic sequences downstream of the insertion (Emery et al. 2002, 2000; Hantzopoulos et al. 1989; Li and Emery 2008; Li et al. 2009).

Shorter versions of cHS4 have been developed which retain the insulator activity of the full length element (Aker et al. 2007; Arumugam et al. 2009). Here we modified the MLV vector pLNHX by

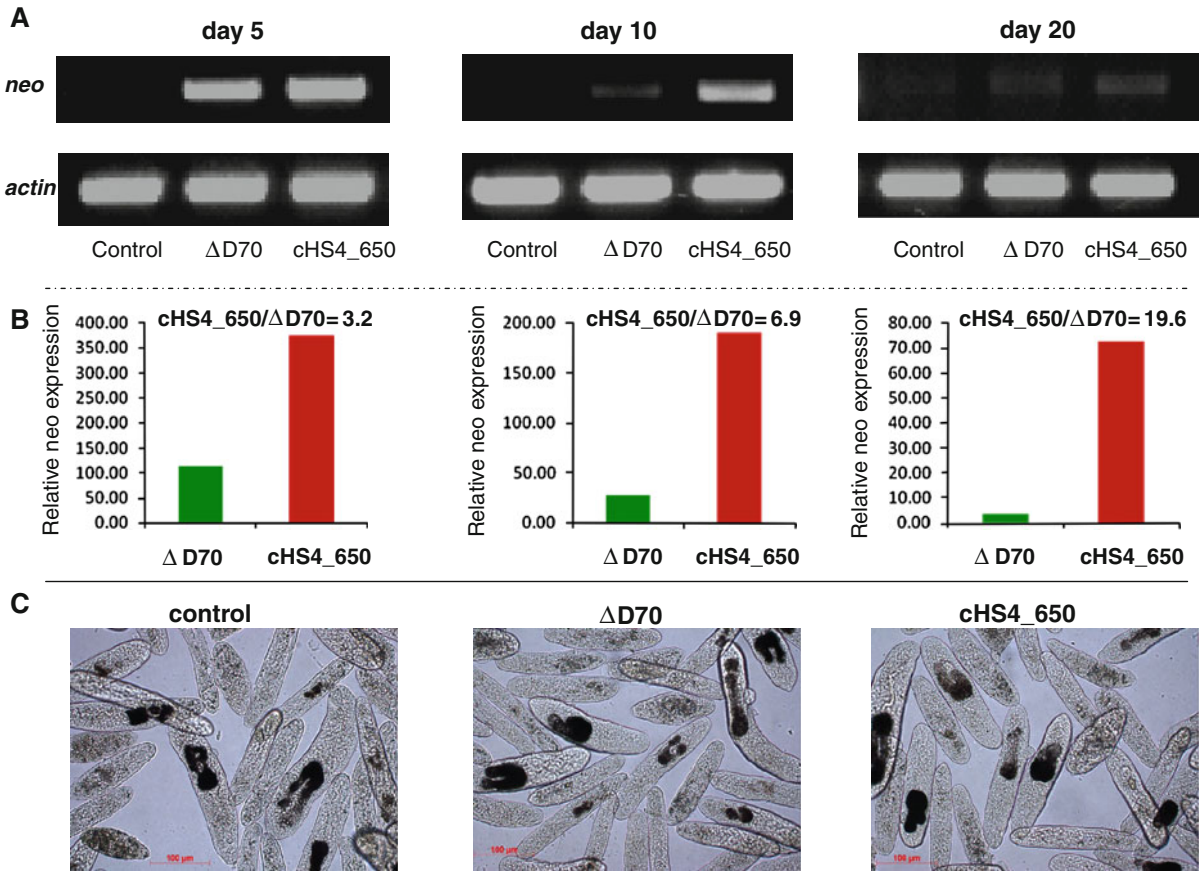

Fig. 3 Expression of *neo* transgene protected from silencing in schistosomules transduced with cHS4 insulated gammaretrovirus virions. **a** Analysis by end-point RT-PCR of *neo* expression in schistosomules 5, 10 and 20 days after exposure to pseudotyped virions where expression levels of *neo* and actin (control for integrity of RNA) were visualized in ethidium-stained agarose gel; control, RNA from age-matched schistosomules not exposed to various; ΔD70, schistosomules exposed to pLNHX_ΔD70 virions; cHS4_650, schistosomules exposed to pLNHX_cHS4_650 virions, in which the chromatin insulator cHS4 fragment flanks the *neo* transgene. **b** Analysis by quantitative RT-PCR of the *neo* expression in schistosomules exposed to pLNHX_ΔD70 virions (*green bars*) or to pLNHX_cHS4_650 virions (*red bars*) for 5, 10 and 20 days as indicated in **a**. Ratio between the relative transgene expression detected in schistosomules transfected with the insulated and non-insulated virions is shown on the *top* of each *panel*. (Note that different *Y*-axis *scales* were used at each time point). **c** Micrographs of schistosomules not exposed to virions (control) or exposed 10 days previously to pLNHX_ΔD70 (ΔD70) or pLNHX_cHS4_650 (cHS4_650) virions. *Bars* = 100 μm. (Color figure online)

insertion of a shortened version of cHS4, comprising 250 bp of the 5′ core region of cHS4 fused to 400 bp of the 3′ region of cHS4. The 650 bp element was inserted into the U3 domain of retroviral 3′-LTR, resulting in replication and transfer to the 5′-LTR bp during reverse transcription (Emery et al. 2000; Hantzopoulos et al. 1989). Relative copy numbers of the transgene were found to be comparable in insulated and control virus transduced schistosomes. By contrast, reporter *neo* transgene expression observed in schistosomules transduced with insulated virions was from two to 20 fold higher in parasites carrying the cHS4 chromatin insulator, flanking both sides of the proviral transgene. Therefore, the stronger *neo* expression detected in the schistosomes transduced by the insulated retrovirus can be explained by a stronger promoter activity (because the transgene was insulated) rather than a higher genome density of integrated proviral transgenes. The increase of *neo* expression in schistosomes transduced with insulated virions may also result from the reduction of position effects which allowed for homogeneity of transgene expression from integrated provirus. In any event, this ratio is similar to the two–tenfold increase in transgene

expression in cHS4 insulated virus compared to un-insulated counterparts seen with short forms of cHS4 in mammalian cells (Arumugam et al. 2007; Emery et al. 2002). On the other hand, there appeared to be down regulation of *neo* expression from both insulated and non-insulated transgenes in the worms over the 20 days during which the transgenic schistosomules were observed. Clearly, however, the cHS4 insulator slowed this process. The general decline in transgene expression may relate to developmental regulation of the promoter, in this case the 5′-LTR of the transgene retrovirus. Alternatively, other transcriptional silencing mechanisms, not efficiently protected against by cHS4, might be at play.

It has been determined that cHS4 is active in a spectrum of metazoans, including vertebrate and insect species (Chung et al. 1993; Emery et al. 2000; Rincon-Arano et al. 2007; Sekkali et al. 2008; Taboit-Dameron et al. 1999). Moreover, this chromatin insulator has been employed in gammaretrovirus, lentivirus, piggyBac transposon, and phiC31 integration system vectors (Aker et al. 2007; Arumugam et al. 2007; Emery et al. 2000; Evans-Galea et al. 2007; Jakobsson et al. 2004; Nishiumi et al. 2009; Rivella et al. 2000; Sarkar et al. 2006). Functional analyses of cHS4 have identified CTCF and USF-1/2 motifs in the proximal 250 bp of cHS4, termed the 'core' region that includes five protein binding sites revealed by DNase I foot printing, which provide enhancer blocking activity and reduce position effects (Bell et al. 1999; Chung et al. 1997; Recillas-Targa et al. 2002). The CTCF binding site footprint II (FII) is necessary and sufficient for enhancer blocking. The binding of USF (upstream stimulatory factor) proteins to footprint FIV is an essential component of its barrier activity (West et al. 2004). USF recruits histone modification complexes and is critical for maintenance of a chromatin barrier (Huang et al. 2007; West et al. 2004). The barrier function of an insulator couples high histone acetylation levels with specific protection of promoter DNA from methylation (Mutskov et al. 2002). Protection from DNA methylation is separable from other insulator activities and is mapped to three transcription factor binding sites occupied by the zinc finger protein VEZF1, a novel chromatin barrier protein and a candidate factor for the protection of promoters from DNA methylation (Dickson et al. 2010). The present study provides the first report of the cHS4 insulator protecting transgene expression in schistosomes; indeed we unaware of investigations of cHS4 in any Lophotrochozoan species. This is notable since the Lophotrochozoan assemblage of phyla includes about half of known metazoan species. Although, the insulator activity of cHS4 on transgene activity has been demonstrated to relate to chromatin modification and enhancer blocking, the mechanism by which cHS4 protects transgene silencing in schistosomes remains to be determined. Database searches indicated the existence of homologues of insulator proteins CTCF, USF and VEZF1 in *S. mansoni* which may support the activity of cHS4 in schistosome chromatin. It would also be of interest to locate schistosome, or other Lophotrochozoan, insulators and to compare their performance with that of cHS4 in these transgenic parasites.

In conclusion, we determined that the prototypic chromatin insulator cHS4 protected transgene expression in schistosomes from silencing. We consider this to be a noteworthy advance in development of vectors that can deliver sustained transgene expression which will enhance the prospects for functional genomics approaches for schistosomes utilizing VSVG-MLV transgenesis. The insulated MLV encoding *neo* can be tested for neomycin/G418 selection of transgenic schistosomes and for germ line transgenesis, given that *neo* is active in *C. elegans* and facilitates the selection of transgenic worms on G418 (Giordano-Santini and Dupuy 2011; Giordano-Santini et al. 2010). More specifically, this short form of cHS4 can be included in gain-of-function vectors to protect expression of reporter genes in schistosome including *neo* and luciferase, into loss-of-function vector-RNAi constructs to ensure expression of transgene hairpin RNAs (Ayuk et al. 2011; Tchoubrieva et al. 2010), and in vectors targeting the schistosome germ line (see Mann et al. 2011). This addition to the nascent functional genomics toolkit for schistosomes will facilitate identification of new interventions targeting this neglected tropical disease pathogen.

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B. EVALUACIÓN DE LA TRANSFECCIÓN ESTABLE DE CÉLULAS GERMINALES

B.1. Estudio de la viabilidad para generar modificaciones genéticas en huevos de *Schistosoma mansoni*

ARTICULO: La interferencia de ARN contra leucin-aminopeptidasa inhibe la eclosión de huevos de *Schistosoma mansoni*

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RNA interference targeting leucine aminopeptidase blocks hatching of *Schistosoma mansoni* eggs

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ABSTRACT

Schistosoma mansoni leucine aminopeptidase (LAP) is thought to play a central role in hatching of the miracidium from the schistosome egg. We identified two discrete LAPs genes in the *S. mansoni* genome, and their orthologs in *S. japonicum*. The similarities in sequence and exon/intron structure of the two genes, LAP1 and LAP2, suggest that they arose by gene duplication and that this occurred before separation of the mansoni and japonicum lineages. The *Sm*LAP1 and *Sm*LAP2 genes have different expression patterns in diverse stages of the cycle; whereas both are equally expressed in the blood dwelling stages (schistosomules and adult), *Sm*LAP2 expression was higher in free living larval (miracidia) and in parasitic intra-snail (sporocysts) stages. We investigated the role of each enzyme in hatching of schistosome eggs and the early stages of schistosome development by RNA interference (RNAi). Using RNAi, we observed marked and specific reduction of mRNAs, along with a loss of exopeptidase activity in soluble parasite extracts against the diagnostic substrate L-leucine-7-amido-4-methylcoumarin hydroxide. Strikingly, knockdown of either *Sm*LAP1 or *Sm*LAP2, or both together, was accompanied by ≥80% inhibition of hatching of schistosome eggs showing that both enzymes are important to the escape of miracidia from the egg. The methods employed here refine the utility of RNAi for functional genomics studies in helminth parasites and confirm these can be used to identify potential drug targets, in this case schistosome aminopeptidases.

1. Introduction

Schistosomiasis is endemic to 76 developing countries and affects an estimated 200 million people in impoverished areas of Africa, Central and South America and East Asia [1,2]. The adult blood flukes locate in mesenteric blood vessels (in *Schistosoma mansoni*, *S. japonicum*, *S. intercalatum* and *S. mekongi*) or the venous plexuses around the urinary bladder (in *S. haematobium*) and after sexual maturation release several hundreds to thousands of eggs a day during their long lifespan (up to 10 years). While some of these eggs reach the lumen of the intestines or the bladder, depending of the infecting species, and are discharged with the feces or urine, many are either trapped in the host tissues or transported by the circulation to the liver and other distant sites [3]. The host inflammatory reaction to the secretions produced by the embryonated eggs leads to formation of granulomas, which evolve in severe cases to portal fibrosis, hepato-splenomegaly and gastrointestinal varices [2–4].

The complement of antigens secreted by schistosome eggs includes protease activities [5–8]. Leucine aminopeptidase (LAP) activity was detected in schistosome eggs more than 30 years ago [9,10], and infected humans and rodents elicit antibody responses to the enzyme [11–13]. Histochemical studies localized the enzyme to eggs and surrounding liver tissue in experimentally infected mice [14]. Recent proteomic and glycomic approaches identified a LAP amongst a complex suite of proteins and glycoproteins within the schistosome egg [15–17]. Because bestatin, an inhibitor of aminopeptidase activity, markedly decreased the escape of miracidia from the eggs it was suggested that LAP activity was critical to the hatching process [18]. LAP activity was also demonstrated in the gut of adult schistosomes by McCarthy et al. [19] who considered that the enzymes play a downstream role in catabolism of ingested hemoglobin and serum proteins.

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In this study, we identified a second LAP gene in *S. mansoni* that is distinct but closely related to the transcript characterized previously [19]. Both LAP enzymes, here termed *Sm*LAP1 [19] and *Sm*LAP2, belong to Clan MF, family M17 metalloproteases [20] according to the MEROPS classification which includes aminopeptidases from archaea, bacteria and eukaryotes that function in protein turnover and biogenesis [21]. The two LAPs exhibit distinct temporal expression profiles during the life cycle of the schistosome. They are both expressed at the egg stage, although *Sm*Lap2 has an elevated expression in eggs and intra-snail stages. Using RNA interference (RNAi) targeting both *Sm*LAP1 and *Sm*LAP2, we now present evidence that proves the two aminopeptidases are required for hatching of eggs of *S. mansoni*.

2. Materials and methods

2.1. Schistosome eggs

Eggs of *S. mansoni* were isolated from livers of mice 7 or 8 weeks after experimental infection, as described [22]. Briefly, three to five livers were chopped finely with a scalpel blade, and then blended to a smooth consistency in 50 ml of phosphate-buffered saline, pH 7.4 (PBS), 5 ml of 0.5% clostridial collagenase (Sigma) and 500 μl of polymyxin B. The mixture was incubated with shaking at 37 °C overnight, after which the contents were subjected to centrifugation at 400 × *g* for 5 min. The supernatant was discarded and the pellet resuspended in 50 ml PBS. This wash procedure was repeated twice more, with the exception that after the final centrifugation the pellet was resuspended into 25 ml of PBS. The resuspended mixture was passed sequentially through 250 and 150 μm sieves. The filtrate was centrifuged at 400 × *g* for 5 min, the supernatant discarded and the pellet resuspended in 3 ml of PBS. This was applied to a column of Percoll, prepared by mixing 8 ml of Percoll (GE Healthcare Bio-Science AB) with 32 ml of 0.25 M sucrose in a 50 ml tube. The tube was centrifuged at 800 × *g* for 10 min. Liver cells that remained on the top of the Percoll were removed with a Pasteur pipette. The schistosome eggs, which pelleted tightly at the bottom of the tube, were washed three times with PBS and any residual liver cells were removed by discarding the supernatants. Further purification of eggs was achieved by resuspension in 0.5 ml of PBS and application on to a second Percoll column, prepared by mixing 2.5 ml of Percoll with 7.5 ml of 0.25 M sucrose in a 15 ml polypropylene tube. The eggs were pelleted and then washed as before. The eggs were resuspended in 6 ml of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 100 U of penicillin and streptomycin (Invitrogen, Carlsbad, CA), split into 2 ml aliquots in a six-well plate and cultured at 37 °C, under 5% CO_2. Other developmental stages of the *S. mansoni* were obtained as described [23,24].

2.2. Bioinformatics characterization of schistosome leucine aminopeptidase

The protein sequences of *S. mansoni* (P91803) [19] and *Fasciola hepatica* (AY064459) [25] LAPs described previously were used as queries for blast searches using the Omniblast interface available at the *S. mansoni* Genome Project page at the Sanger Institute, www.sanger.ac.uk/cgi-bin/blast/submitblast/s_mansoni/omni. Only two genomic regions produced significant hits with the queries, one being identical to the P91803 query termed *Sm*LAP1 (GeneDB Smp_030000), and the other termed *Sm*LAP2 (Smp_083870). The genomic scaffolds containing the hits were visualized in the genome browser available at the *S. mansoni* GeneDB, www.genedb.org/genedb/smansoni/. A similar search was performed on *S. japonicum* using as database the 95,389 contigs of the assembly release 2 available from the Shanghai Centre for Life Sciences & Biotechnology Information, http://lifecenter.sgst.cn/sjschistosoma/en/schistosomaDispatch.do?disName=intro. The contigs or scaffolds including the putative LAP loci from both species were retrieved and further analysed using Artemis; synteny was evaluated by comparison using the Artemis Comparison Tool, ACT [26]. The deduced amino acid sequences of the *S. mansoni* and *S. japonicum* LAPs were aligned with reference sequences (accessions provided in figure legends) using ClustalX [27]. A bootstrapped neighbor joining tree was generated based on the more conserved carboxy terminal domain, using the aminopeptidase A from *Escherichia coli* as outgroup. Based on the sequences of *Sm*LAP1 and *Sm*LAP2 appropriate specific primers for their amplification were generated (see below for sequences).

2.3. Leucine aminopeptidase expression analysis

RNA was extracted from different *S. mansoni* stages using the RNAqueus-Micro Kit (Ambion, Austin, TX). Residual DNA contaminating the RNA was removed by DNase digestion (TurboDNase, Ambion). cDNAs were synthesized using 300 ng of total RNA, using the iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA). Leucine aminopeptidase 1 (*S. mansoni* Gene DB Smp_030000) (*Sm*LAP1) and leucine aminopeptidase 2 (*S. mansoni* Gene DB Smp_083870) (*Sm*LAP2) expression was investigated by RT-PCR using gene specific primers (F: 5′-CAT TCC CAG AAC ATC CAT CAA A-3′; R: 5′-CAT TAT CCA TCA CAG CCG TGA A-3′ spanning 1020 bp for *Sm*LAP1, and F: 5′-TAC AAG AAG CAT CAC AGG TGA A-3′; R: 5′-CAG AGT AGC GAT TGT CAT TAG TC-3′ spanning 1070 bp, for *Sm*LAP2). *Sm*LAP1 and *Sm*LAP2 were confirmed by cloning the PCR products in pCR4.0 TOPO TA vector (Invitrogen, CA, USA) and sequencing. Expression of *S. mansoni* actin gene (GenBank U19945) [28,29] was used as an internal control using the primers, F: 5′-CAG TGT TCC CTT CCA TCG TT-3′; R: 5′-GGA CAG GGT GTT CTT CTG GA-3′, spanning 224 bp. PCR conditions included an initial denaturation at 94 °C for 30 s followed by 35 cycles of 30 s at 94 °C, 60 s at 52 °C, 90 s at 72 °C and a final extension at 72 °C for 10 min. Images of PCR products in ethidium-stained gels were documented using a Versadoc imaging system and Quantity One software (BioRad). Densitometric measurements of ethidium-stained PCR product bands were obtained using Image J, http://rsbweb.nih.gov/ij/.

2.4. Synthesis of dsRNA

dsRNAs were transcribed *in vitro*, from template PCR products, using gene specific primers tailed with the T7 promoter sequence. Irrelevant control firefly luciferase dsRNA (dsLUC) template encoding the full length 1672 kb was generated using the pGL3-basic plasmid (Promega, Madison, WI) as template and primers F: 5′-*TAA TAC GAC TCA CTA TAG GG* T GCG CCC GCG AAC GAC ATT TA-3′ (T7 promoter residues indicated in italics); R: 5′-*TAA TAC GAC TCA CTA TAG GGG* CAA CCG CTT CCC CGA CTT CCT TA-3′. *Sm*LAP1 and *Sm*LAP2 clones were employed for synthesis of *Sm*LAP1 dsRNA (dsLAP1) and *Sm*LAP2 dsRNA (dsLAP2) using primers that included the T7 promoter sequence (F: 5′-*TAA TAC GAC TCA CTA TAG GG*A CGA ACA TTA GCA CGA GAT ATT-3′; R: 5′-*TAA TAC GAC TCA CTA TAG GG*C ATA ACC ATT CTA CCT TCA GCA-3′ spanning coding DNA positions 543–1077 for dsLAP1; and F: 5′-*TAA TAC GAC TCA CTA TAG GG*G CTG AAG TCC TGG GTT GGT T-3′; R: 5′-*TAA TAC GAC TCA CTA TAG GG*C CAT TCG ACC TTC AGC ATC A-3′ spanning coding DNA positions 502–1117 for dsLAP2). dsRNA was synthesized and purified using the Megascript RNAi kit (Ambion). dsRNAs were precipitated with one volume of 5 M ammonium acetate and 2.5 volumes of 95% ethanol after which the RNA pellet was dissolved in water. Integrity of the dsRNAs was verified by non-denaturing 1% agarose gel electrophoresis,

and concentration and purity determined with a spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE).

2.5. Delivery of dsRNA

Immediately after the isolation, eggs were cultured in 1,000 μl of DMEM complete media as described above, at 37 °C under 5% CO_2 in 24 wells plate (~5000 eggs per well). The eggs were soaked in 20 μg/ml of either ds*Sm*LAP1, ds*Sm*LAP2, ds*Sm*LAP1 and ds*Sm*LAP2 together, or dsLUC (the latter serving as a control, irrelevant dsRNA). Another group of eggs was cultured without dsRNA, as a mock-treatment control. The media and the dsRNA were changed every other day, with frequent microscopic examination to identify visual phenotypic effects. The eggs were harvested after 7 days in culture, for subsequent RNA and protease activity analyses, and in addition an aliquot of eggs from each treatment group was induced to hatch (below).

2.6. RNAi effects

2.6.1. Hatching of eggs

Eggs from each group were washed three times in 1× PBS, added to 500 μl of distilled water and exposed to bright light. Micrographs of the cultured eggs were taken, 30 and 60 min later, using an inverted microscope fitted with a digital camera. The ratio of non-hatched to total eggs in each group was determined from visual inspection of the images; 80–100 eggs were inspected in each treatment group in each experiment. Another group of eggs was incubated with the aminopeptidase inhibitor, bestatin, following the protocol of Xu and Dresden [18]. Briefly, 5 μl of bestatin (Sigma–Aldrich, St. Louis, MO) (10 mg/ml in DMSO) was added to the eggs (~5000 eggs) in 45 μl of 0.2 M of NaCl, and the eggs incubated for 60 min at 23 °C. Subsequently, 950 μl dH_2O was added to the eggs to stimulate hatching of the eggs, after which hatching was monitored and documented as above. In addition, a control group of eggs without the inhibitor bestatin, but with the DMSO carrier, was included. These assays were repeated twice.

2.6.2. RNA level: gene expression analysis

Eggs were harvested 7 days after treatment with dsRNA. Total RNA was extracted using the RNAqueus-Micro Kit (Ambion). Residual DNA contaminating RNA preparations was removed by DNase digestion (TurboDNase, Ambion). cDNAs were synthesized using 10-fold serial dilutions of egg RNA, starting with 100 ng RNA, using the iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA). Primers targeting the LAP and actin transcripts, and PCR cycle conditions, are described above.

2.6.3. Protein level: LAP enzyme activity assay

Soluble protein extracts from eggs were prepared by sonication-induced lysis (5 × 5 s bursts on ice, output control value 3, model W-220F Sonicator, Heat Systems-Ultrasonics, Inc., Plainview, New York) in 100 mM glycine, pH 8.5 containing 1 mM $MnCl_2$. After centrifugation of the lysate for 10 min at 4 °C at 14,000 rpm, the supernatant was employed as soluble schistosome eggs extract. A total of 2 μg of soluble protein from each group was added in triplicate to a 100 μl reaction mixture of 100 mM glycine, pH 8.5 containing 1 mM $MnCl_2$ and the fluorogenic substrate L-leucine-7-amido-4-methylcoumarin hydroxide (Bachem) at a final substrate concentration of 50 μM, and incubated at 37 °C for 30 min. Fluorescence from substrate hydrolysis was measured in a fluorometer (BioTek Synergy HT, Winooski, VT) at 430 nm with excitation at 360 nm. To confirm that LAP activity was being measured, the egg extracts were pre-incubated for 10 min with 50 μM concentration of the broad-spectrum aminopeptidase inhibitor, bestatin (Sigma–Aldrich), prior to the addition of the substrate. Protein concentrations of soluble egg extracts were determined using the bicinchoninic acid assay (BCA kit, Pierce, Rockford, IL).

2.7. Statistical analysis

Levels of statistical significance among and between treatments were determined using Analysis of Variance (ANOVA) and Student's *t*-test. *P*-values of ≤0.05 were considered to be significant.

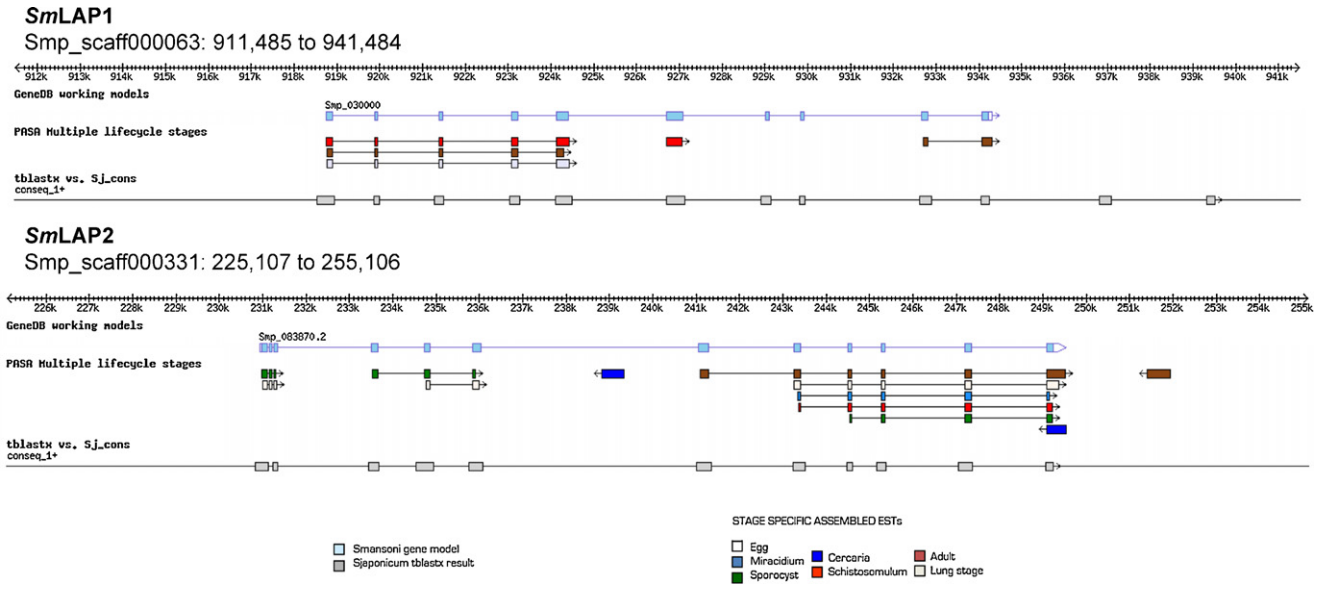

Fig. 1. A schematic view of the genome loci encoding the *Schistosoma mansoni* leucine aminopeptidase (LAP) genes. The genome browser view, obtained from www.genedb.org/schistosoma/, indicates the position of the exons of *Sm*LAP1 and *Sm*LAP2 (coding portions in light blue boxes, 3′ untranslated regions in white boxes). High scoring regions on tblastx comparisons against the *S. japonicum* genome draft are indicated (grey boxes). Stage specific assembled ESTs (color coded) mapped with the PASA package [60] on the genomic sequence and their orientations are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

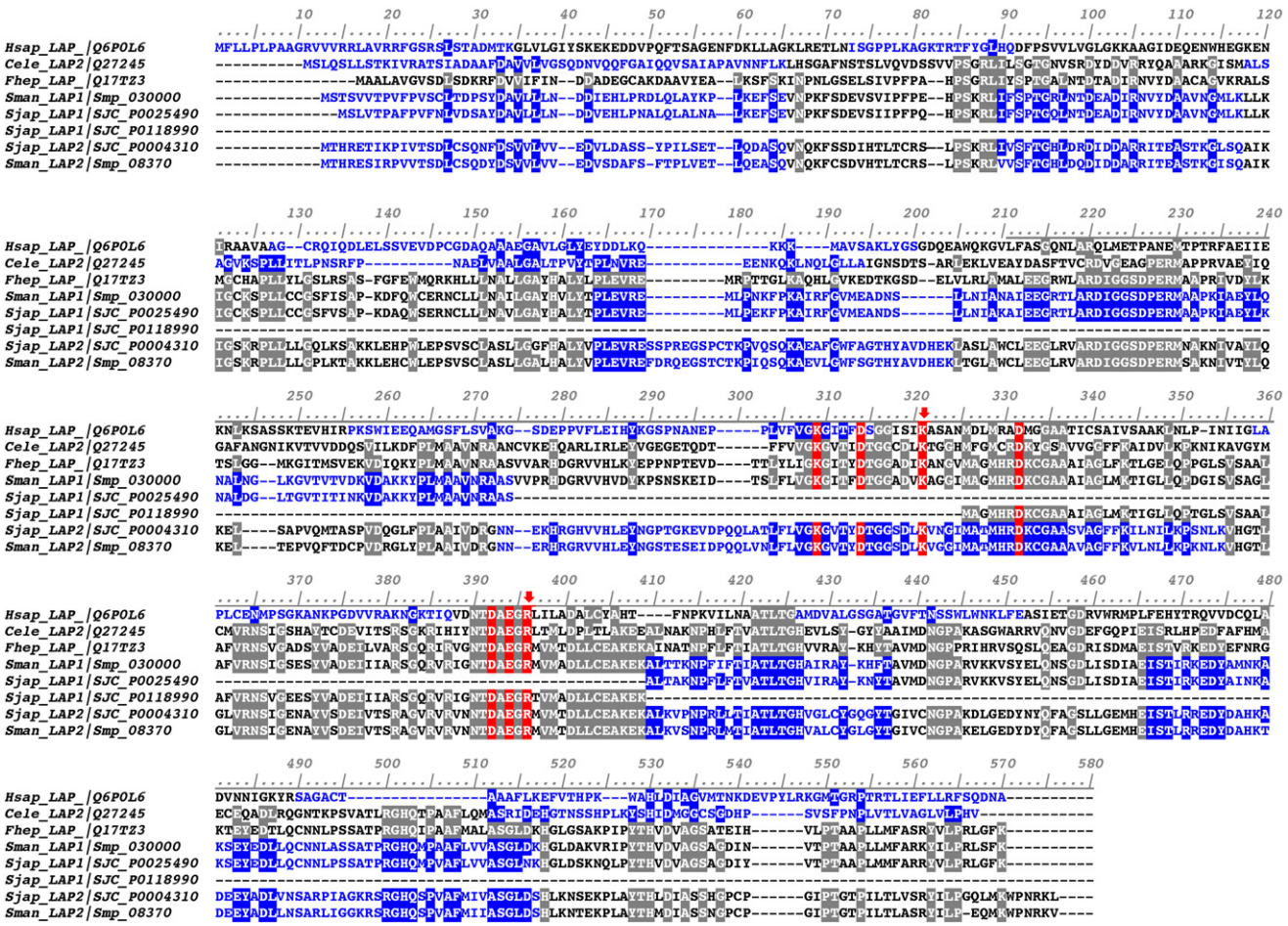

Fig. 2. Alignment of amino acid sequences of leucine aminopeptidases. Exon alternation in genomic sequences is indicated by alternation of blue and black type. Conserved regions are shown as shaded blocks. Residues that participate in coordination of metal ions are indicated in red and those involved in the catalysis are arrowed. (By way of reference, residues Asp 289, Asp 367 and Glu 369 [*Sm*LAP1 numbering] are involved in the coordination of a zinc atom, while Asp 289, Lys 284, Asp 307 and Glu 369 bind a second zinc ion. Lys 296 and Arg 371 are also involved in catalysis.) The conserved carboxy terminal domain is indicated by underlining in the alignment positions. The reference sequences are from *Fasciola hepatica* (accession Q17TZ3) *Caenorhabditis elegans* (Q27245) and *Homo sapiens* (Q6P0L6). The schistosome identifiers correspond to those provided in the genome assembly 4 of *Schistosoma mansoni* and assembly 2 of *Schistosoma japonicum*. The two contigs comprising *Sj*LAP1 are included. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3. Results

3.1. Two leucine aminopeptidase genes in Schistosoma mansoni genome

To investigate the complement of LAP genes in the genome of *S. mansoni*, we searched the draft genome (version 4.0) available at the Sanger Institute, a seven-fold coverage that likely includes all the protein encoding genes of the schistosome. The search identified two genes; one corresponding to the cDNA reported previously, which we refer to now as *Sm*LAP1, encoding a protease of 523 deduced amino acid residues, and a novel gene, *Sm*LAP2, of 544 deduced amino acid residues (Fig. 1). A similar search of the draft of the *S. japonicum* genome located the putative orthologs of both genes. No other M17 aminopeptidase-like sequences were found in the schistosome genomes. An alignment of the deduced amino acid sequences of the *S. mansoni* and *S. japonicum* LAPs 1 and 2 with closely related enzymes from *Homo sapiens*, *F. hepatica* and *Caenorhabditis elegans* is presented in Fig. 2. The *Sm*LAP1 and *Sm*LAP2 sequences were 49% identical and 63% similar at the amino acid level, while *Sm*LAP1 and *Sj*LAP1 were 89% identical and 95% similar, *Sm*LAP2 and *Sj*LAP2 were 88% identical and 93% similar. *Sm*LAP1 and *Sm*LAP2 were 20–21% identical to human LAP, respectively. LAPs exhibit a two domain structure, a less conserved N-terminal domain and a conserved C-terminal domain that contains the catalytic residues. Residues Asp 289, Asp 367 and Glu 369 (*Sm*LAP1 numbering) are involved in the coordination of a zinc atom, while Asp 289, Lys 284, Asp 307 and Glu 369 bind a second zinc ion. Lys 296 and Arg 371 are also involved in the catalytic mechanism by acting as an electrophile and proton donor, respectively.

The *Sm*LAP1 gene spans 15.5 kb in the center of scaffold 000063 (1959 Mb), and is structured in 10 exons, encoding 523 deduced amino acid residues, and includes a short 101 bp 3′UTR. The *Sm*LAP2 gene covers 18.59 kb of scaffold 000331 (779 kb) comprising 12 exons, consisting of a 28 bp 5′UTR, 1635 bases of the CDS (544 deduced amino acid residues) and a 293 bp 3′UTR (Fig. 1). A similar search in the draft of the *S. japonicum* genome allowed us to identify the putative orthologs of both genes. *Sj*LAP2 maps to contig SJC_C002437 (included in the scaffold SJC_000012). The coding sequence (SJC_P0004310) is also organized in 12 exons identical to those of *Sm*LAP2, although the introns are bigger, giving the whole gene a length of 21.9 kb. There are two contigs with high homology to *Sm*LAP1; scaffold SJC_000149 covers the full length of the gene, comprising exons 1–5 and 7–10. The sixth exon is missing due to a sequencing gap included in the scaffold; a different small contig (SJC_010679, 3.4 kb) contains the last portion of the missing exon (see Figs. S1 and S2). No other relevant hits to LAPs were

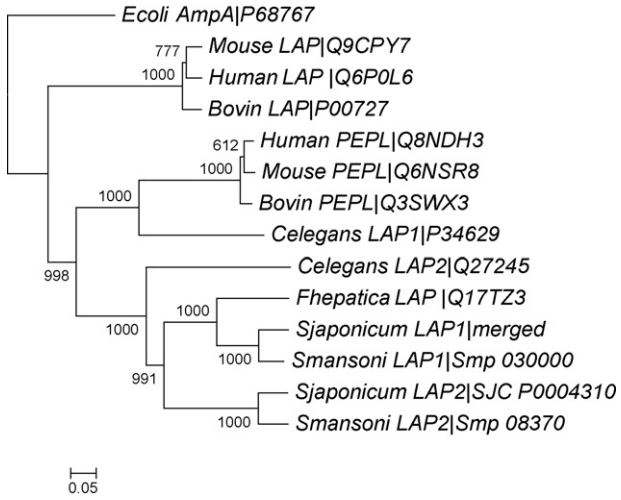

Fig. 3. Phylogenetic tree reveals relationships between schistosome leucine aminopeptidases and other family M17 LAP enzymes. Neighbor joining tree based on the conserved carboxy terminal domain of selected proteins. The aminopeptidase A from *Escherichia coli* served as the outgroup. Bootstrap values supporting the major clusters are indicated. Branch names indicate species or common name along with Uniprot accessions.

detected in the *S. japonicum* genome. The comparison of the coding sequences of the schistosome LAP1 and LAP2 genes indicated that they are closely related. The difference in their exonic structure is restricted to the fifth and sixth exons of LAP1 that are split in two exons each respectively in LAP2 genes. This structural arrangement is conserved between both schistosome species, suggesting that the homologues emerged by a duplication event previous to separation of the *mansoni* and *japonicum* species (Figs. 1 and 2). Phylogenetic analysis confirmed that schistosome LAP1 and LAP2 were closely related, and constituted a separate clade with other flatworm LAPs and *C. elegans* LAP2, discrete from the vertebrate LAPs (Fig. 3).

3.2. Differential developmental expression of the leucine aminopeptidases

Information provided at the schistosome GeneDB of ESTs mapping to the genomic sequences indicated that both *Sm*LAP1 and *Sm*LAP2 genes were expressed in different stages of the schistosome developmental cycle, with *Sm*LAP1 exhibiting a more restricted expression (Fig. 1). In order to confirm this observation and assess the expression level of the two LAPs during development of *S. mansoni*, we designed gene specific primes to clone *Sm*LAP1 and *Sm*LAP2. 300 ng of total RNA from different stages were used to perform RT-PCR using gene specific primers. Actin gene expression was used as a control. The identity of the PCR products as *Sm*LAP1 and *Sm*LAP2 was confirmed by nucleotide sequencing (not shown). Interestingly, whereas both LAPs are equally expressed in the blood dwelling stages (schistosomules and adult) *Sm*LAP2 expression was higher in free living larval (miracidia) and in parasitic intra-snail (sporocysts) stages (Fig. 4A). There were no apparent differences in the expression of the two LAPs in adult male and female blood flukes, while the absence of expression in cercarial stage was in concordance with the global reduction of gene expression in cercariae reported by Jolly et al. [30] (Fig. 4A). There were no apparent sex-related differences between male and female worms or combined adults for any of the three genes, including actin. Densitometric measurements of the relative levels of *Sm*LAP1 and *Sm*LAP2 measured across the different bands in Fig. 4A using actin as normalization control are shown in Fig. 4B. Interestingly, ESTs mapping data suggest that only a reverse transcript is found in this stage, which may indicate that anti-sense transcripts are involved in silencing gene expression in cercariae (Fig. 1).

3.3. RNAi targeting LAPs inhibited hatching of schistosome eggs

We employed RNAi to investigate the putative function of LAP activity in the egg hatching process. We soaked cultured eggs with ds*Sm*LAP1 RNA, ds*Sm*LAP2 RNA, or both, or with bestatin. Control eggs were cultured without dsRNAs or bestatin, while other eggs treated with bestatin served as a positive control. In addition, eggs treated with double stranded RNA specific for firefly luciferase (dsLUC), a gene not present in the schistosome genome, were included as a dsRNA treatment control. After 7 days in culture (during which time no phenotypic differences were apparent among the treatment groups), eggs were washed in PBS, transferred into water at 23 °C under bright light, and photographed 60 min later to quantify how many miracidia had hatched from the eggs. Representative micrographs of eggs during the hatching process 1 h after incubation in water are presented in Fig. 5, panel A. More specifically, soaking in dsRNAs targeting *Sm*LAP1, *Sm*LAP2, or both together, markedly inhibited the hatching process (Fig. 5A, panels C–E), quantified as the ratio between hatched eggs over total egg number. In contrast, the number of empty shells found in the control without treatment or in the irrelevant dsRNA treated group was significantly higher than in the other treated groups (Fig. 5A, panels A and B). Bestatin blocked hatching to similar levels of the dsRNA targeting the LAPs (Fig. 5A, panel F). Specifically, bestatin and the dsRNAs targeting LAPs inhibited hatching by ~80% compared to the control treatment groups and the group with the irrelevant dsRNA Luc (Fig. 5B). ANOVA demonstrated significant differences among the groups ($P < 0.01$); moreover, significantly fewer eggs hatched in each of the dsLAP groups and the bestatin group compared with the controls ($P < 0.01$). The inhibition experiments were repeated twice, with similar results (not shown).

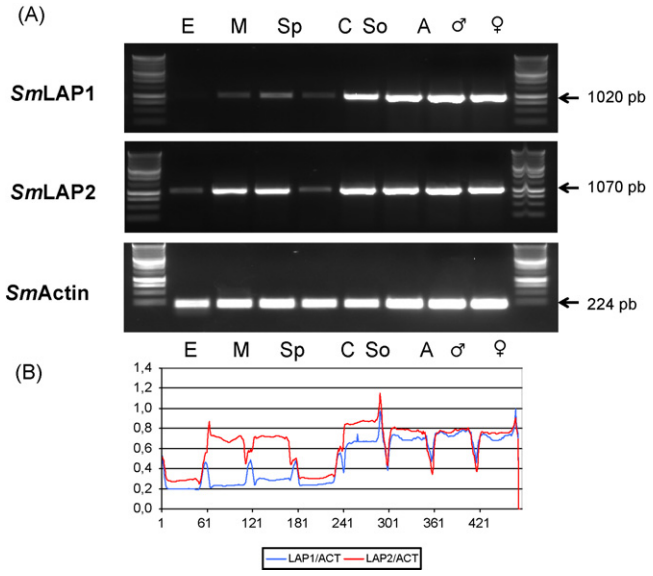

Fig. 4. Developmental stage specific expression of leucine aminopeptidases (LAP) in *Schistosoma mansoni*. (Panel A) cDNAs from eggs (E), miracidia (M), sporocysts (Sp), cercariae (C), schistosomules (So), mix sex adults (A), male adults (♂) and female adults (♀) were employed as templates for PCRs using primers specific for the *Sm*LAP1, *Sm*LAP2 and *S. mansoni* actin genes. (Panel B) Densitometric measurements (arbitrary units) across the different bands and relative levels of *Sm*LAP1 (blue) and *Sm*LAP2 (red) compared with levels for actin presented in panel A. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

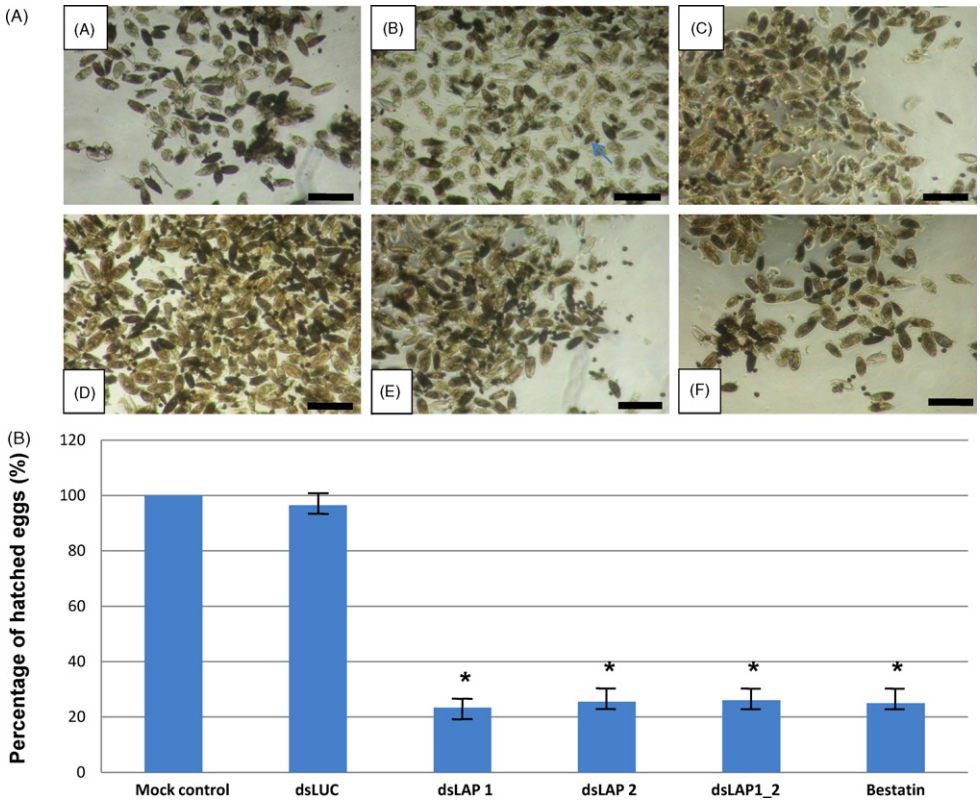

Fig. 5. Knockdown of egg hatching by RNA interference in *Schistosoma mansoni*. Hatching of eggs was blocked by dsRNA against *Sm*LAP1, *Sm*LAP2, both together, and bestatin. (A) Representative micrographs of eggs during the hatching process, 1 h after incubation in water. A: mock control, B: dsLUC treated eggs, C: dsLAP1 treated eggs, D: dsLAP2 treated eggs, E: eggs treated with dsLAP1 and dsLAP2, F: bestatin treated eggs. Bar, 200 μm. (B) Percentage of hatched eggs: 100%, control eggs. Statistically significant differences were evident among the dsLAP1 group, dsLAP2 group, dsLAP1 + dsLAP2 group, bestatin group and the controls mock and irrelevant dsRNA. The bars indicate standard deviation of mean for two experiments, and asterisks indicate significant differences ($P < 0.01$).

3.4. Transcript levels and LAP activity in eggs knocked down by RNAi

To corroborate the egg-hatch data, we investigated leucine aminopeptidase transcription and translation in RNAi-treated and non-treated eggs. Total RNA isolated from representatives of the control and experimental treated groups was 10-fold diluted serially and these dilutions were employed as the template for RT-PCR. As illustrated in Fig. 6B, the inhibitory effect mediated by the dsRNA was specific for *Sm*LAP1 and *Sm*LAP2, since transcripts for schistosome actin (*Sm*Actin) appeared to be unaffected by dsLAP1, and dsLAP2 treatment, and demonstrated that RNAi successfully mediated blocking of LAP transcription. We observed that expression of *Sm*LAP1 was elevated in the experiment presented in Fig. 6 in comparison to the study described in Fig. 4. Whereas the reason for these differences in expression in these two discrete trials was not clear, within each experiment there was consistent evidence of specific RNAi-mediated knockdown of *Sm*LAP1.

We then assessed enzyme activity in soluble extracts of the eggs by employing the LAP-diagnostic peptide H-Leu-NHMec as the substrate, although it should be noted that this substrate would not distinguish between the two enzymes. LAP activity in eggs harvested 7 days after exposure to the dsRNAs was reduced in *Sm*LAP-dsRNA treated eggs with relative fluorescence units (RLUs)/μg schistosome protein of 14,585 RLUs/μg for the mock-treatment control, 20,841 RLUs/μg for the dsRNA irrelevant control (dsLUC), 8939 RLUs/μg for the dsLAP1 treated group, 9355 RLUs/μg for the dsLAP2 treated group, and 11,118 RLUs/μg for the dsLAP1 and dsLAP2 treated group (ANOVA, $F = 149$, df = 9, $P < 0.01$) between the dsLAP treated groups and both controls). The extracts of *Sm*LAP-dsLAP1 treated eggs exhibited only 42% as much activity as the dsRNA irrelevant control group ($P < 0.01$); similarly, while extracts of *Sm*LAP-dsLAP2-treated eggs exhibited only 44% as much activity as the control dsLUC treated eggs ($P < 0.01$) (Fig. 6A). The effect at RNA level in the irrelevant control is accompanied by an increase in LAP enzyme activity as indicated in Fig. 6A. A similar effect has been seen in other dsRNA silencing experiments by our group and others at RNA or protein levels [31–33]. Furthermore, this effect is clearly seen only when a mock group is included in addition to an irrelevant control, but not when the irrelevant is the only control present as happens usually in several reports.

Fig. 6A and B revealed a discrepancy with respect to the semi-quantitative RT-PCR and the enzyme assay. In the eggs treated with both *Sm*LAP1 and *Sm*LAP2 dsRNAs there was no product in RT-PCR compared to either *Sm*LAP1 or *Sm*LAP2 treatments alone. In contrast, when the enzyme activity was assayed, eggs treated with both *Sm*LAP1 and *Sm*LAP2 dsRNAs showed higher activity than the singly treated samples. One explanation of these contrasting findings is that H-Leu-NHMec may have been hydrolyzed by other, as yet uncharacterized, aminopeptidases or other proteases in the egg extracts.

4. Discussion

Leucyl aminopeptidases are hexameric metalloproteases found in archaea, bacteria and eukaryotes where they perform diverse physiological functions, including protein turnover, redox status, MHC antigen presentation, site specific recombination, bacterial transcription, and pathogenesis of cataract formation and other pathologies. In higher eukaryotes they are involved in post-proteasome processing of peptides, generation and/or elimination

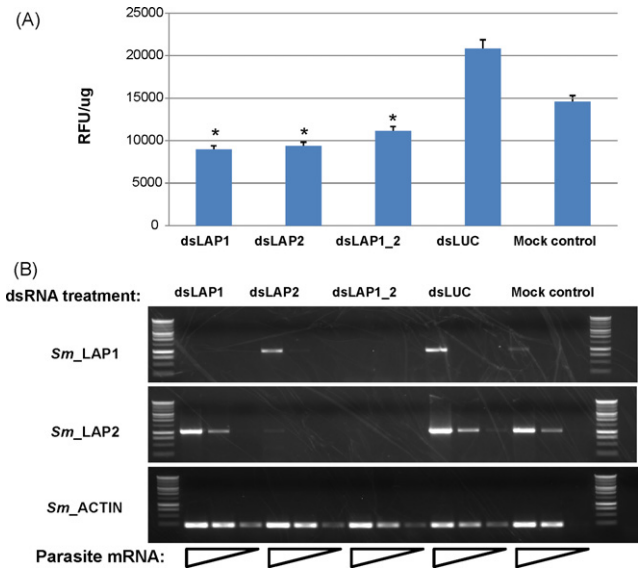

Fig. 6. RNAi targeting schistosome leucine aminopeptidases results in statistically significant knockdown of protease activities and mRNA levels. (Panel A) Leucine aminopeptidase (LAP) activity assay performed using the diagnostic substrate, L-leucine-7-amido-4-methylcoumarin hydroxide. LAP activity in relative fluorescence units per microgram (RLU/μg) of soluble schistosome protein presented on *Y*-axis. Groups of eggs were treated with double stranded RNA targeting *Sm*LAP1, *Sm*LAP2, both, firefly luciferase, and not treated with dsRNA (mock control). (Panel B) Semi-quantitative RT-PCR using LAP specific primers and actin as an internal control. Schistosome egg cDNAs were synthesized from serial dilutions of total RNAs (100, 10 and 1 ng) isolated from treatment group of eggs, soaked in doubled strand RNA targeting *Sm*LAP1, *Sm*LAP2, both, firefly luciferase, and not treated with dsRNA (mock control). The bars show the standard deviations for two experiments. Asterisks indicate significant differences ($P < 0.01$) between treated groups and both controls groups. The difference between the mock control and the irrelevant dsRNA control also was significant ($P < 0.01$).

of active biopeptides, and regulation of signal transduction pathways [34,35].

Dresden and co-workers [15,26,27] demonstrated that LAP like activity is present in schistosome eggs and was associated with the hatching of miracidia from eggs, since this activity was detected in hatching fluid and emergence of miracidia was inhibited by bestatin [18,36,37]. Bestatin, the dipeptide *N*-((2*S*,3*R*)-3-amino-2-hydroxy-4-phenyl butanyl) L-leucine is an antibiotic of microbial origin with broad-spectrum activity against leucine aminopeptidases, aminopeptidases B and N and other metalloproteases [38–40]. Our results supports the findings of Dresden and co-workers, and indeed have extended them by employing RNAi in parallel with bestatin to definitively show that blocking schistosome LAP activity prevents the hatching process. Thus, soaking schistosome eggs in dsRNA targeting *Sm*LAP1, *Sm*LAP2 or both of them markedly blocked egg hatching and emergence of miracidia. The magnitude of egg-hatch inhibition was similar in range and amplitude to that obtained with bestatin [18]. The visible phenotype was accompanied by an almost complete ablation of the corresponding mRNA, and a marked diminution of LAP activity in soluble extracts of eggs against the diagnostic substrate H-Leu-NHMec. The disappearance of the mRNA indicated that the effect was due to mRNA degradation as expected for RNAi. Furthermore, although knockdown of either *Sm*LAP1 or *Sm*LAP2 elicited almost identical reductions in hatching, these were specific since the RNAi selectively reduced expression of the targeted LAP with no effect on the other aminopeptidase. The RNAi effects indicated that the LAPs either play a direct role in hatching or they provide metabolites essential for the process.

Although leucine aminopeptidase activity had been known from the egg and adult stages of both *S. mansoni* and *S. japonicum* [17,19], there was little information on the genes that encoded this activity. The availability of the draft genome of *S. mansoni*, the current draft of which has >7-fold genome coverage, facilitated targeted bioinformatics searches, from which we recognized a second M17 family leucine aminopeptidase gene, discrete from the original cDNA reported by McCarthy et al. [19]. A similar search in the genome draft of *S. japonicum* predicted two related genes, orthologous to those found in *S. mansoni*. The genomic structure of both schistosome LAP1 and LAP2 genes was similar, including the length, conservation of intron positions, and number of exons. The difference between LAP1 and LAP2 exon count, 10 and 12 exons respectively, could be accounted for by a split in two of the fifth and sixth exons of LAP1 in two exons each in LAP2. These data, the sequence similarity between them and the phylogenetic analysis support the hypothesis that they emerged by a duplication event. Because the genomic structure was conserved between the two schistosome species, it seems likely that the duplication occurred before speciation of Asian (*japonicum*) and African (*mansoni*) and schistosomes [41]. Whereas the two genes are closely related, the central position of both genes in the corresponding scaffolds of the *S. mansoni* genome draft and the differences in the surrounding genes make it unlikely that they are located in tandem in the genome. Furthermore, the phylogenetic analysis confirmed that trematode LAPs are dissimilar to the mammalian homologues which constitute separate clades (Fig. 3) [25].

The *S. mansoni* LAPs exhibited differences, compared to each other, in developmental expression as measured directly by RT-PCR, which was supported by the presence of stage specific ESTs. While both enzymes have similar strong expression in the mammalian stages, only LAP2 was expressed strongly in miracidia and sporocysts. Only modest expression of both LAPs was observed in cercariae, a situation that might be related to the anti-sense transcripts in ESTs from cercariae, and consistent with a general reduction of gene expression in this developmental stage [30]. Expression levels of the LAPs in the eggs were not high as indicated by RT-PCR signals despite their critical importance in hatching [14,18,36,37]. *Sm*LAP1 occurs in the gut of the adult fluke where it has been ascribed a role in terminal digestion of hemoglobin and other ingested host proteins [19].

LAPs have been characterized in several other trematodes, including *Paragonimus westermani* [42] and *F. hepatica* [25,43], although a role in egg hatching has not been determined definitely in these flukes. However, the relevance of LAP as a potential target for control of trematodiases at large was highlighted by experimental immunization of sheep with native *F. hepatica* LAP, which delivered 89% protection against challenge infection along with reduced disease [43,44]. Moreover, recombinant *F. hepatica* LAP tested as a vaccine antigen in rabbits elicited a strong antibody response and 78% reduction in worm burden after challenge [25]. RNAi directed towards LAP of *F. hepatica* induced specific mRNA degradation in juvenile *F. hepatica*, although morphological phenotypes were not apparent [33]. LAPs appear to play important roles in development and fecundity in other invertebrates. In the gastrointestinal nematode *Haemonchus contortus*, LAP has been associated with molting and egg hatching since both processes can be blocked with bestatin and induced by Zn^{2+} [45–49]. LAP activity is present in other parasitic nematodes [50,51]. In the free living *C. elegans* null mutants of LAP1 show reduced growth and retarded egg-laying [52]. Inhibition by RNAi of the cytosolic LAP of the tick *Haemaphysalis longicornis* resulted in a delay in oviposition and reduction in egg biomass [53].

The present investigation confirmed long held views that exopeptidase activity ascribable to leucine aminopeptidase was critical to the hatching of schistosome eggs. We have now definitively shown – using RNA interference targeting two schistosome LAP genes – that schistosome LAP appears to be necessary for hatching of the schistosome egg. Schistosome eggs are fully embry-

onated as when passed in the feces or urine and, as a consequence, miracidia can hatch immediately from the egg in the environment [54]. Osmotic changes that occur as the egg enters freshwater are the primary cue for emergence of the miracidia from the eggshell [55–57]. Downstream of the osmotic changes, physiological and molecular events leading to hatching include calcium ion fluxes and the activity of LAP [56–58]. Indeed, release of LAP immediately follows activation of *S. mansoni* eggs [18,57]. One possibility is that schistosome LAPs participate in the digestion of components of the inner membrane of the egg in the hatching process [54]. The identity of the natural substrate(s) for LAP from eggs has not been reported. However, potential roles for LAP include scission of the outer envelope-shell boundary, autolysis of the inner envelope, or/and degradation of proteins in the lacunae [54].

It has been reported that TGF-β signaling plays a major role in the embryogenesis of *S. mansoni*; RNAi-mediated knockdown of SmInAct expression in eggs aborts egg development [59]. Our data suggest that LAP acts at a later stage of development since the effect is seen on the emergence of fully differentiated miracidia. Immunological responses to eggs trapped in the livers and other organs of infected persons represent the root cause of the pathology of schistosomiasis. Accordingly, a deeper understanding of the role of LAP in development of eggs as well as in hatching, as of the receptors and other components of the schistosome egg [16,58] can be expected to have broad implications for novel treatment and control of this significant neglected tropical disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2009.05.002.

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B.2. Evaluación de la transfección y transgénesis en huevos de *S. mansoni*

ARTICULO: La electroporación facilita la introducción de transgenes reporteros y viriones en huevos de *Schistosoma*

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(* Igual contribución al trabajo)

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PLoS NEGLECTED TROPICAL DISEASES

Electroporation Facilitates Introduction of Reporter Transgenes and Virions into Schistosome Eggs

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Abstract

Background: The schistosome egg represents an attractive developmental stage at which to target transgenes because of the high ratio of germ to somatic cells, because the transgene might be propagated and amplified by infecting snails with the miracidia hatched from treated eggs, and because eggs can be readily obtained from experimentally infected rodents.

Methods/Findings: We investigated the utility of square wave electroporation to deliver transgenes and other macromolecules including fluorescent (Cy3) short interference (si) RNA molecules, messenger RNAs, and virions into eggs of *Schistosoma mansoni*. First, eggs were incubated in Cy3-labeled siRNA with and without square wave electroporation. Cy3-signals were detected by fluorescence microscopy in eggs and miracidia hatched from treated eggs. Second, electroporation was employed to introduce mRNA encoding firefly luciferase into eggs. Luciferase activity was detected three hours later, whereas luciferase was not evident in eggs soaked in the mRNA. Third, schistosome eggs were exposed to Moloney murine leukemia virus virions (MLV) pseudotyped with vesicular stomatitis virus glycoprotein (VSVG). Proviral transgenes were detected by PCR in genomic DNA from miracidia hatched from virion-exposed eggs, indicating the presence of transgenes in larval schistosomes that had been either soaked or electroporated. However, quantitative PCR (qPCR) analysis determined that electroporation of virions resulted in 2–3 times as many copies of provirus in these schistosomes compared to soaking alone. In addition, relative qPCR indicated a copy number for the proviral luciferase transgene of ~20 copies for 100 copies of a representative single copy endogenous gene (encoding cathepsin D).

Conclusions: Square wave electroporation facilitates introduction of transgenes into the schistosome egg. Electroporation was more effective for the transduction of eggs with pseudotyped MLV than simply soaking the eggs in virions. These findings underscore the potential of targeting the schistosome egg for germ line transgenesis.

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Introduction

Advances in molecular genetics and immunology hold the promise to control the spread of schistosomiasis and to combat the morbidity and mortality associated with this neglected tropical disease [1]. Currently, control of schistosomiasis largely relies on chemotherapy with praziquantel, but its widespread use has led to concerns about development of drug resistance [2]. Schistosomes have comparatively large genomes, estimated at 398 megabase pairs (MB) for the haploid genome of *Schistosoma japonicum* [3] and 363 MB for *S. mansoni* [4]. Schistosome genes are arrayed on seven pairs of autosomes and one pair of sex chromosomes. *S. haematobium*, the other major schistosome species parasitizing humans probably has a genome of similar size, based on similarity of the karyotypes [5]. The schistosome genomes are the first to be published from among the Lophotrochozoa, an assemblage that includes about half of all animal phyla [6]. Analysis of the genomes revealed the presence of ~13,000 protein-encoding genes, about 40% repetitive sequence content (retrotransposons, etc.), pervasive domain structure reduction, complex signal transduction and sensory pathways, proliferation of mini-exons, curious intron size distribution, large numbers of protease encoding genes, and other remarkable features [3,4,7].

Despite this abundance of sequence data, functional analysis of potential target genes will not be possible until reliable methods for reverse genetics in schistosomes become available. Transformation and gene manipulation in schistosomes have been reviewed recently (e.g., [8–10]. Schistosomes are large, multicellular

Author Summary

The genome sequences of two of the three major species of schistosomes are now available. Molecular tools are needed to determine the importance of these new genes. With this in mind, we investigated introduction of reporter transgenes into schistosome eggs, with the longer-term aim of manipulation of schistosome genes and gene functions. The egg is a desirable developmental stage for genome manipulation, not least because it contains apparently accessible germ cells. Introduction of transgenes into the germ cells of schistosome eggs might result in transgenic schistosomes. However, the egg is surrounded by a thick shell which might block access to entry of transgenes. We cultured eggs in the presence of three types of reporter transgenes of increasing molecular size, and in addition we tried to produce transient holes in the eggs by electroporation to investigate whether the transgenes would more easily enter the eggs. Electroporation of eggs appeared to allow entry of two larger types of transgenes into cultured schistosome eggs, messenger RNA encoding firefly luciferase, and retroviral virions. We anticipate that this approach, electroporation of transgenes into schistosome eggs, will facilitate genetic manipulation of schistosomes for investigating the importance of schistosome genes and gene products as new intervention targets.

eukaryotes, and though aceolomate, they possess complex organ systems including a blind gut with absorptive and secretory functions, well developed muscles, nervous tissues with complex sensory systems (like eyespots), and separate sexes with complex female and male reproductive tissues. The blood stage forms are covered by a syncytial tegument that is bordered at the parasite-host interface with a double lipid bilayer. Furthermore, the developmental stages differ dramatically in appearance and structure, cell numbers, ratio of germ to soma, and morphology. All these features pose challenges for genetic manipulation, and especially for germ line transgenesis. However, genetic manipulation and germ line transgenesis are worthwhile goals because they would facilitate a deep understanding of the molecular biology of schistosomes, roles of molecules in host-parasite interaction and, ultimately, to identify gene products that could be targeted/disrupted with drugs or vaccines.

Although the entire developmental cycle of the human schistosomes cannot be maintained *in vitro*, laboratory maintenance of the developmental cycles of all three human schistosomes can be accomplished using rodents as the mammalian host and the intermediate host snails [11,12]. In addition, several developmental stages including mammalian and molluskan parasitic stages can be maintained *in vitro* (see [13]). Schistosome eggs can be obtained from livers of experimentally infected rodents, and miracidia obtained from these eggs are infectious for the intermediate host [14,15]. In addition, the eggs can be maintained *in vitro* for at least one week and retain viability [16–19].

The schistosome egg represents an advantageous developmental stage of the schistosome at which to target transgenes because of its availability from experimentally infected rodents, high ratio of germ to somatic cells and because miracidia hatching from eggs can be employed to infect snails and propagate the developmental cycle. On the other hand, the developing miracidium is enclosed within an electron-dense, environmentally resistant egg shell [20–22]. Here we explored the introduction of transgenes and other macromolecules into eggs of *S. mansoni* by square wave electroporation. Square wave electroporation was more efficient than soaking alone for transduction of schistosome eggs by messenger RNA encoding luciferase and by pseudotyped retrovirus virions.

Materials and Methods

Developmental stages of *S. mansoni*

Mice infected with the NMRI (Puerto Rican) strain of *Schistosoma mansoni* were supplied by Dr. Fred Lewis, Biomedical Research Institute, Rockville, MD. Both adult worms and eggs were recovered from infected mice, as described [14], using a protocol approved by the Institutional Animal Case and Use Committee of The George Washington University. Eggs recovered from mouse livers were cultured for up to seven days at 37°C under 5% CO_2 in air in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U of penicillin and streptomycin [13,19]. These eggs were washed three times in phosphate buffered saline, pH 7.4 (PBS) before exposure to Cy3-labeled siRNAs, mRNA, or virions. Virion-exposed eggs were transferred to sterile water under bright light to induce egg hatching and release of miracidia [13]. Miracidia were harvested from hatching eggs every 30 min for two hours. In some PCR based experiments, genomic DNAs, isolated from virion transduced sporocysts, and known to contain integrated proviral transgenes [23] were included as positive controls.

Exposure of eggs to Cy3-labeled siRNA

S. mansoni eggs were either electroporated and soaked in non-coding Cy3-labeled siRNAs (Silencer Cy3-Labeled Negative Control siRNA, catalog no. AM4621, Ambion, Austin, TX) at 50 ng/µl with conditions recommended by Correnti et al. [24], or exposed similarly to Cy3-labeled siRNA without electroporation. Eggs were electroporated in 100 µl of schistosomule wash medium (RPMI 1640 with 200 U/ml penicillin G sulfate, 200 µg/ml streptomycin sulfate, 500 ng/ml amphotericin B, 10 mM HEPES) in 4 mm gap cuvettes with an ElectroSquarePorator ECM830 (BTX, San Diego, CA) using a single square wave pulse of 125 volts for 20 milliseconds. After electroporation, eggs were transferred into complete DMEM at 37°C. Three hours after exposure to Cy3-siRNA, with or without electroporation, eggs were washed in culture medium three times in order to remove the unincorporated Cy3-labeled siRNAs. Thereafter, they were observed under bright and fluorescent light (see below) using a Zeiss Axio Observer A.1 inverted microscope fitted with a digital camera (AxioCam ICc3, Zeiss). The eggs were cultured overnight and additional images collected 18 hours after electroporation and/or soaking. Manipulation of digital images was undertaken with the AxioVision release 4.6.3 software (Zeiss).

Synthesis and delivery of luciferase mRNA

To synthesize firefly luciferase mRNAs (mFLuc), a template was prepared using PCR amplification of the luciferase gene in plasmid pGL3-Basic (Promega, Madison, WI) as described [25]. *In vitro* transcriptions of capped RNAs from DNA templates were accomplished using the mMessage mMachine T7 Ultra kit (Ambion, Austin, TX). Subsequently, ammonium acetate precipitated mFLuc was dissolved in nuclease-free water and quantified by spectrophotometry (ND-1000, NanoDrop Technologies, Wilmington, DE). *S. mansoni* eggs in culture for 48 h after isolation from mouse livers were subjected to electroporation in the presence of, and/or soaked in mFLuc at 100 and 130 ng/µl. Briefly, approximately 1,500–2,500 eggs were subjected to the square wave electroporation in 4 mm gap pathway cuvettes (BTX) in 120 µl of schistosomule wash medium [13,25] using one

20 millisecond pulse of 125 volts. Thereafter, eggs were transferred to complete DMEM at 37°C, cultured for 3 h, or as indicated, washed three times in schistosomule wash medium and then stored as pellets at −80°C. Similar numbers of eggs were also soaked in complete DMEM with mFLuc at 130 ng/µl and maintained in culture for 3 h and washed three times before harvest.

Luciferase activity assay

Luciferase activity in extracts of eggs was monitored using Promega's luciferase assay reagents [25,26]. In brief, eggs were disrupted by sonication (5×5 sec bursts, output cycle 5, Misonix Sonicator 3000) (Misonix, Farmingdale, NY 11735) in 300 µl 1× CCLR lysis buffer (Promega). Aliquots of the egg sonicate (100 µl) were injected into 100 µl luciferin (Promega) at room temperature, mixed, and the relative light units (RLUs) determined ten seconds later at 560 nm in a Sirius tube luminometer (Berthold, Pforzheim, Germany). Duplicate samples were measured, with results presented as the average RLU readings per µg of soluble egg protein. The protein concentration in the soluble fraction of the egg lysate was determined using the bicinchoninic acid assay (BCA kit, Pierce, Rockford, IL). Recombinant firefly luciferase (Promega) was included as a positive control.

Transduction of schistosome eggs with pseudotyped retrovirus

VSVG-pseudotyped virions were produced in GP2-293 cells transfected with plasmid constructs pLNHX-*Sm*ACT-Luc and pVSVG, as described [23]. pLNHX-*Sm*ACT-Luc includes the reporter gene encoding firefly luciferase (FLuc) under control of the actin gene promoter of *S. mansoni* [23,27]. Viral supernatants were incubated with DNase I (New England Biolabs, Ipswich, MA) to remove any contaminating pLNHX plasmids using the method of Bruce et al [28]. After centrifugation, the pellet of concentrated virions was resuspended in Opti-MEM Reduced Serum Medium (Invitrogen). The viral titer was determined with a biological assay using target NIH-3T3 mouse fibroblast cells cultured in the presence of geneticin, as described [23].

Schistosome eggs were cultured in 35-mm tissue culture plates in ~2 ml medium containing ~200 µl of virions (VSVG-MLV) at 6×10^5 colony forming units (cfu)/ml in the presence of 8 µg/ml polybrene (Sigma-Aldrich, St. Louis, MO). Other eggs exposed to the same VSVG-MLV inocula were also subjected to square wave electroporation using methods adapted from Pearce and coworkers [29]; the eggs were electroporated in 4 mm gap cuvettes in 400 µl of schistosomule wash medium and 200 µl of VSVG-MLV virions, using a single 125 V pulse of 20 milliseconds duration, as above. After electroporation, eggs were transferred into culture medium containing 8 µg/ml polybrene. Eighteen hours later, eggs were washed to remove virions and polybrene, and cultured for a further two days before hatching. Subsequently, genomic DNAs were isolated from the miracidia, and the presence or absence of the luciferase proviral transgene was investigated by direct PCR.

In two additional experiments, a quantitative PCR (qPCR) strategy was used to investigate whether electroporation could influence copy number of proviral transgenes. In the first experiment, eggs were cultured for two days before exposure, by electroporation or soaking, to ~200 µl of a virion suspension (VSVG-MLV) at 2×10^4 cfu/ml containing 8 µg/ml polybrene. Eggs were cultured for three days after virion exposure, after which they were transferred water to induce release of miracidia. For the second qPCR experiment, eggs were cultured for three days before exposure by electroporation or soaking to the virion suspension described above, cultured for two more days after virion exposures, and then eggs transferred to water to induce release of miracidia. In both experiments, at one day after exposure to virions, eggs were washed in culture medium to remove virions and polybrene. gDNAs were isolated from the miracidia, and employed as templates for qPCR analysis of transgene copy numbers.

Detection of provirus in transduced schistosomes

Total genomic DNA (gDNA) was isolated from transduced and control untreated developmental stages of schistosomes, including mixed sex adult worms, using the AquaPure system (Bio-Rad, Hercules, CA). In order to investigate the presence of double stranded, proviral transgenes, we employed gDNAs isolated from miracidia hatched from transduced eggs as templates for direct PCR using the firefly luciferase primers 5′-GGAGAGCAACTG-CATAAGG and 5′-AATCTCACGCAGGCAGTTCT (see below). As a positive control for the PCR, we amplified the *S. mansoni* cytochrome oxidase I (*cox 1*) gene (GenBank AF101196, using *cox 1*-specific primers 5′-TGAGTGTCATTTTAGGGTGGTG and 5′-ACAAACCAATGAAAATATCCAAGA) which we have shown previously to be amplified from these kinds of gDNA preparations [27]. In addition, as negative controls, we included templates of gDNA from non-virion exposed adult schistosomes and/or included reactions where water was substituted for gDNA. PCRs were carried out using Master Mix (Promega) reagents, and 35 thermal cycles of 94°C, 1 min, 50°C, 1 min, and 72°C, 2 min. Amplification products were separated by electrophoresis through 1% agarose, stained with ethidium bromide, visualized under UV illumination and digital images captured (Gene-Doc, Bio-Rad). After electrophoresis to determine their sizes, PCR products were Southern blotted onto Zeta-Probe (Bio-Rad) nylon. A ~5.3 kb *Kpn* I fragment pLNHX-*Sm*ACT-Luc (including the luciferase coding sequence) [27] was isolated, labeled with ^{32}P.dCTP by random oligomer priming (RadPrime, Invitrogen) [27] and used as a probe. Southern blots were hybridized at 65°C to the labeled probe for 18 h, washed at high stringency [30], and hybridization signals detected by autoradiography on Biomax film (Kodak).

Real-time quantitative PCR and estimation of transgene copy numbers

Primers were designed with the assistance of Beacon Designer (Premier Biosoft International, Palo Alto, CA) to obtain primer and TaqMan probe sequences targeting the firefly luciferase (FLuc) (from pGL3-Basic, Promega, Madison, WI) and *S. mansoni* cathepsin D (*Sm*CathD) (GenBank U60995) genes, as follows: for FLuc, forward primer: 5′-TGC TCC AAC ACC CCA ACA TC-3′; reverse primer: 5′- ACT TGA CTG GCG ACG TAA TCC-3′; probe: 5′-/56-FAM/ACG CAG GTG TCG CAG GTC TTC C/3IABlk_FQ/-3′; for *Sm*CathD, forward primer : 5′-TGG GCT CAC TGA GTG TAA AGG-3′; reverse primer: 5′-CAT ACC AAG GAT ACC ATC GAA CTT C-3′; probe: 5′-/56-FAM/ ACC CTG GTT GTT GTG TCG CTT CCC/3IABlk_FQ/-3′. Quantitative PCRs were performed in triplicate, using 96-well plates (Bio-Rad), with al denaturation step at 95°C from 3 minutes followed by 40 cycles of 30 sec at 95°C and 30 sec at 55°C, using a thermal cycler (iCycler, Bio-Rad) and a Bio-Rad iQ5 detector to scan the plates in real time. Reactions were carried out in 20 µl volumes with primer-probe sets (FLuc, *Sm*CathD) and Perfecta qPCR FastMix, UNG (Quanta Bioscience, Gaithersburg, MD).

Absolute quantification was undertaken using 250 ng of gDNA samples or copy number standards, i.e. 10-fold serial dilutions of pGL3, from 1.93×10^3 copies to 1.93×10^{10} copies. The exact copy number of each diluted plasmid was calculated through the relationship between the molecular mass of pGL3 and the

Avogadro constant, N_A. Absolute copy number of the luciferase transgene per ng of schistosome gDNA was estimated by interpolation of the sample PCR signals from a standard curve (see [31]).

Relative quantification was performed in order to estimate the transgene copy number in comparison with an endogenous schistosome gene of known copy number [31,32] in particular the single copy number gene, *Sm*CathD. *Sm*CathD encodes the cathepsin D aspartic protease of *S. mansoni* that participates in hemoglobin proteolysis [33]. The PCR efficiencies for the FLuc transgene and the *Sm*CathD gene were estimated by titration analysis [31] to be 99.0% and 97.3%, respectively (not shown). Five 10-fold decreasing serial dilutions starting from 200 ng of gDNA of each sample were used as templates to target the *Sm*CathD and FLuc genes, in different reactions. Estimation of the relative copy number of FLuc was derived from ΔCt values for *Sm*CathD. To calculate ΔCt values, the average of triplicate Ct values generated with the luciferase primers-probe set was subtracted from the average *Sm*CathD Ct values. The copy number ratio between *Sm*CathD and FLuc in each sample was obtained with the equation, $2^{\Delta Ct}$ [31].

Statistical analysis

Statistical differences among and between groups were investigated using analysis of variance (ANOVA) and Student's *t*-test. *P*-values of ≤ 0.05 were considered to be significant.

Results

Cy3-labeled siRNA penetrates into *S. mansoni* eggs

To investigate whether macromolecules could penetrate into *S. mansoni* eggs, cultures of schistosome eggs were incubated in a Cy3-siRNA (~13.8 kDa) with or without concomitant square wave electroporation. Three hours after exposure to Cy3-siRNA, eggs were examined by fluorescence microscopy which revealed diffuse but weak fluorescence within the eggs (not shown). By contrast, by 18 hours after soaking or electroporation with Cy3-siRNA, eggs displayed strong fluorescence including foci of intense fluorescence (Figures 1, 2). Many eggs had hatched, releasing miracidia; the miracidia displayed multiple intense areas of fluorescence, indicating uptake of Cy3-siRNA by the eggs (Figures 1, 2, panels e, f in both). Either soaked or electroporated eggs with Cy3-siRNA and the corresponding hatched miracidia/sporocysts showed intense fluorescence. In contrast, control eggs treated similarly but without Cy3-siRNA, i.e. mock treatment controls, showed no specific fluorescence (Figure S1). Soaked eggs displayed spots of strong fluorescence throughout the eggs (Figure 1, panel d; Figure S2, panel b). Miracidia/sporocysts hatched from the Cy3-siRNA exposed eggs often exhibited intense signals, with large, bright fluorescent foci (Figure 1, panel f). Compared to eggs soaked with Cy3-siRNA, electroporated eggs displayed a more diffuse Cy-3 fluorescence (Figure 2, panel d and Figure S3, panel b), contained within the egg shell.

The miracidia/sporocysts hatched from electroporated eggs displayed intense foci of fluorescence (Figure 2, panel f; Figure S3, panel d), similar to the Cy3-siRNA soaked groups. The signal was distributed throughout the entire larval body, but often with foci of strong fluorescence at the posterior extremity. Lack of fluorescence signal in the ciliated plates shed from the miracidia indicated that the Cy3-siRNA was incorporated into the larvae and was not retained in the surface (Figure S3, panel d). These data indicated that the electroporated Cy3-siRNA entered the eggs, perhaps traversing through the cribriform pores of the eggshell [22], and entered the miracidium within the eggshell. Neither soaked nor electroporated control eggs displayed fluorescence (Figure 1, panel b; Figure 2, panel b).

Reporter gene luciferase mRNA electroporated into schistosome eggs

To investigate whether transgene mRNAs could penetrate schistosome eggs, we soaked and/or electroporated cultured eggs in firefly luciferase mRNA (mFLuc) (~512 kDa). More specifically, after two days in culture, 1,500–2,500 eggs were soaked or electroporated with 130 ng/μl of mFLuc; the eggs were collected three hours later. Luciferase activity was measured, with relative luminescence units (RLUs/μg) normalized per μg of soluble protein extracted from the eggs. Significant luciferase activity was detected in the mFLuc electroporated group compared with the others ($P<0.05$) (Figure 3A). By contrast, no significant differences were apparent among the other treatment groups. Because significant luciferase activity was observed only in eggs electroporated with mFLuc, we investigated the influence of increasing concentrations of mFLuc, 0 ng/μl, 100 ng/μl, and 130 ng/μl, and in eggs harvested three hours after electroporation. Significant luciferase activity was observed in homogenates of the transformed eggs with all three concentrations of mFLuc whereas untreated worms show negligible activity ($P<0.05$) (Figure 3B). Since mRNAs usually exhibit short half lives *in vivo*, we also examined luciferase activity at 30 hours after electroporation of the eggs with 130 ng/μl, as an indirect measure of mFLuc stability. Little or no luciferase was detected at 30 hours after electroporation (Figure 3B). Collectively, these findings indicated that square wave electroporation efficiently delivered exogenous nucleic acids into the eggs of *S. mansoni*.

Schistosome eggs transduced by pseudotyped retroviral virions

Schistosome eggs were electroporated and/or soaked in the presence of VSVG pseudotyped pLNHX-*Sm*ACT-Luc virions. One to three days later, eggs were incubated in sterile water to induce hatching of miracidia from the virion exposed eggs. We investigated whether these miracidia from eggs exposed to virions had been transduced by the retrovirus. Direct PCR analysis of gDNA isolated from miracidia from transduced eggs was employed to detect the presence of proviral retrovirus (schematic of predicted transgene provirus presented as Figure 4 A). As the positive control for the experiment, a 589 bp fragment of the reporter transgene encoding luciferase was amplified from gDNA from sporocysts known (from our previous studies [23,34]) to contain integrated proviral transgenes *luc* (Figure 4B, lane 1). Likewise the *cox 1* signal of 294 bp was amplified from the sporocyst gDNA (lane 2), indicating the integrity of the PCR. Furthermore, the *luc* transgene was also detected in miracidia from eggs that were either electroporated (lane 3) or soaked (lane 5) in pLNHX-*Sm*ACT-Luc virions. The control *cox 1* gene fragment of 294 bp also was amplified from these gDNAs, verifying the integrity of the templates (Figure 4B, lanes 4, 6). No FLuc gene specific amplification was seen using template gDNA from control worms not exposed to virions (lane 7) whereas the target 294 bp region of the *cox 1* gene was amplified (lane 8) from this control gDNA. The identity of PCR products as specific for the FLuc transgene was confirmed by Southern hybridization analysis to a labeled pLNHX-*Sm*ACT-Luc/*Kpn* I gene probe (Figure 4A). (These PCR findings demonstrated the presence of proviral transgenes within the treated larvae. We anticipate that many of the proviral transgenes had integrated into schistosome chromo-

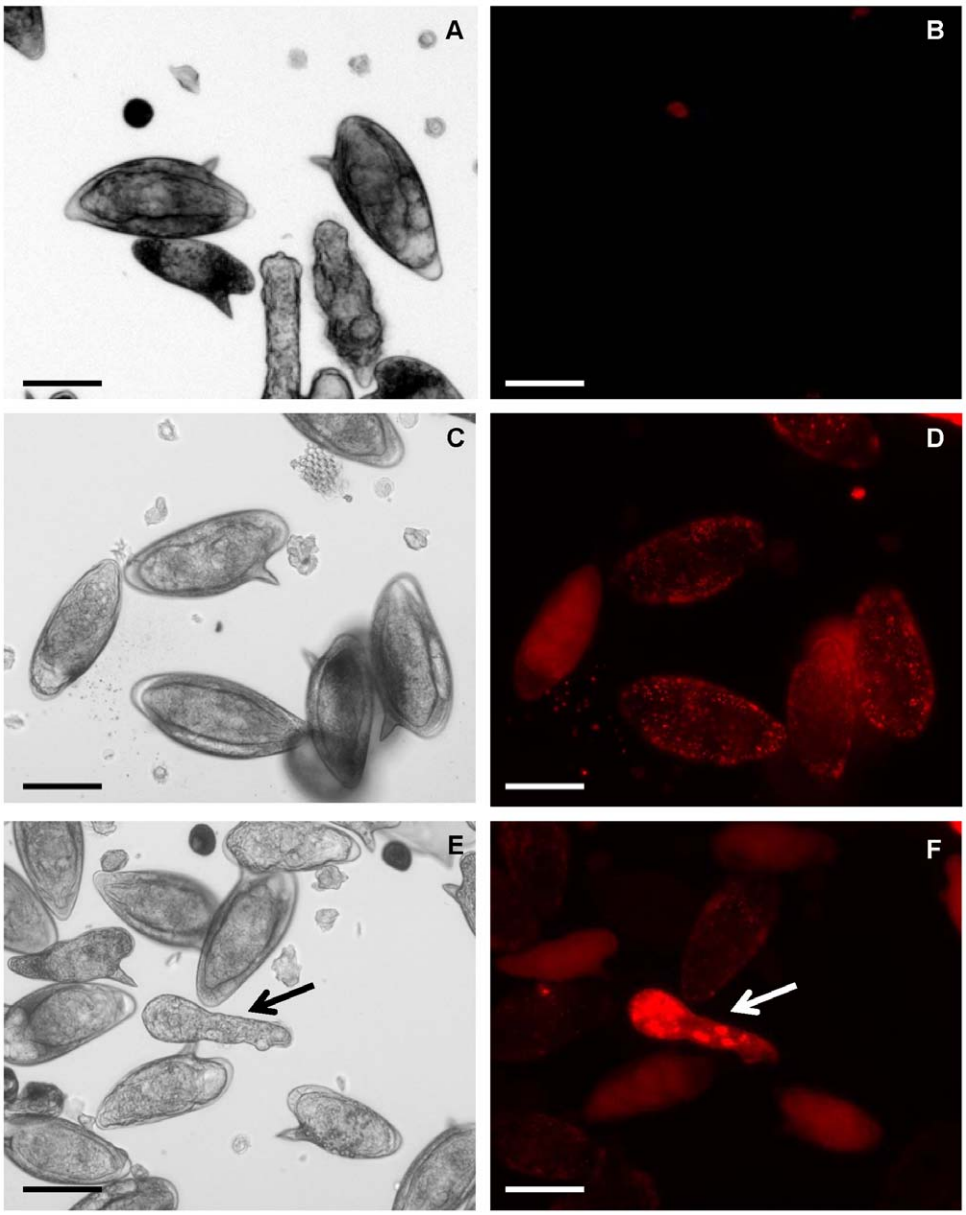

Figure 1. Fluorescent labeled short interfering RNA enters cultured eggs of Schistosoma mansoni. Representative images of schistosome eggs and miracidia 24 hours after soaking in Cy3-siRNA; bright field, upper panels, fluorescence, lower panels. Eggs were soaked in medium containing 50 ng/μl of Cy3-siRNA. No Cy3-siRNA treatment control (A, B), Cy3-siRNA treated, fluorescent eggs (C, D) and fluorescent eggs and a miracidium/sporocyst (arrow in E, F). Scale bar, 50μm.
doi:10.1371/journal.pntd.0000593.g001

somes, based on our earlier findings [27]. Whether or not the provirus had actually integrated or remained as non-integrated provirus does not negate the finding that electroporation was more efficient than soaking for transduction of schistosome eggs (below). However, Southern hybridization analysis of gDNA of a representative group of virion exposed eggs/miracidia indicated that proviral transgenes had integrated into the schistosome chromosomes (not shown).

It was noteworthy that miracidia hatched from the eggs soaked in pseudotyped virions did not appear to have lost vitality because of virion exposure. By contrast, many miracidia that hatched from electroporated eggs were less active; their movement was sluggish compared to miracidia from soaked eggs. Also, many eggs failed to hatch after electroporation (data not shown).

Retroviral transduction of schistosome eggs facilitated by electroporation

Quantitative PCR (qPCR) was employed to determine the copy number of the proviral luciferase transgene in gDNAs from miracidia hatched from virion-exposed eggs. Methods yielding both absolute and relative quantification were used. Figure 5 summarizes the results from two related experiments. Eggs that had been in culture for 48 h (experiment no. 1) and eggs in culture for 72 h (experiment no. 2) were electroporated or soaked with pseudotyped MLV. Three days (no. 1) and two days (no. 2) later, gDNA was isolated from miracidia hatched from the eggs and assayed for the presence of the luciferase transgene by qPCR. In the first, about three times as many copies, and in the second experiment, more than twice as many

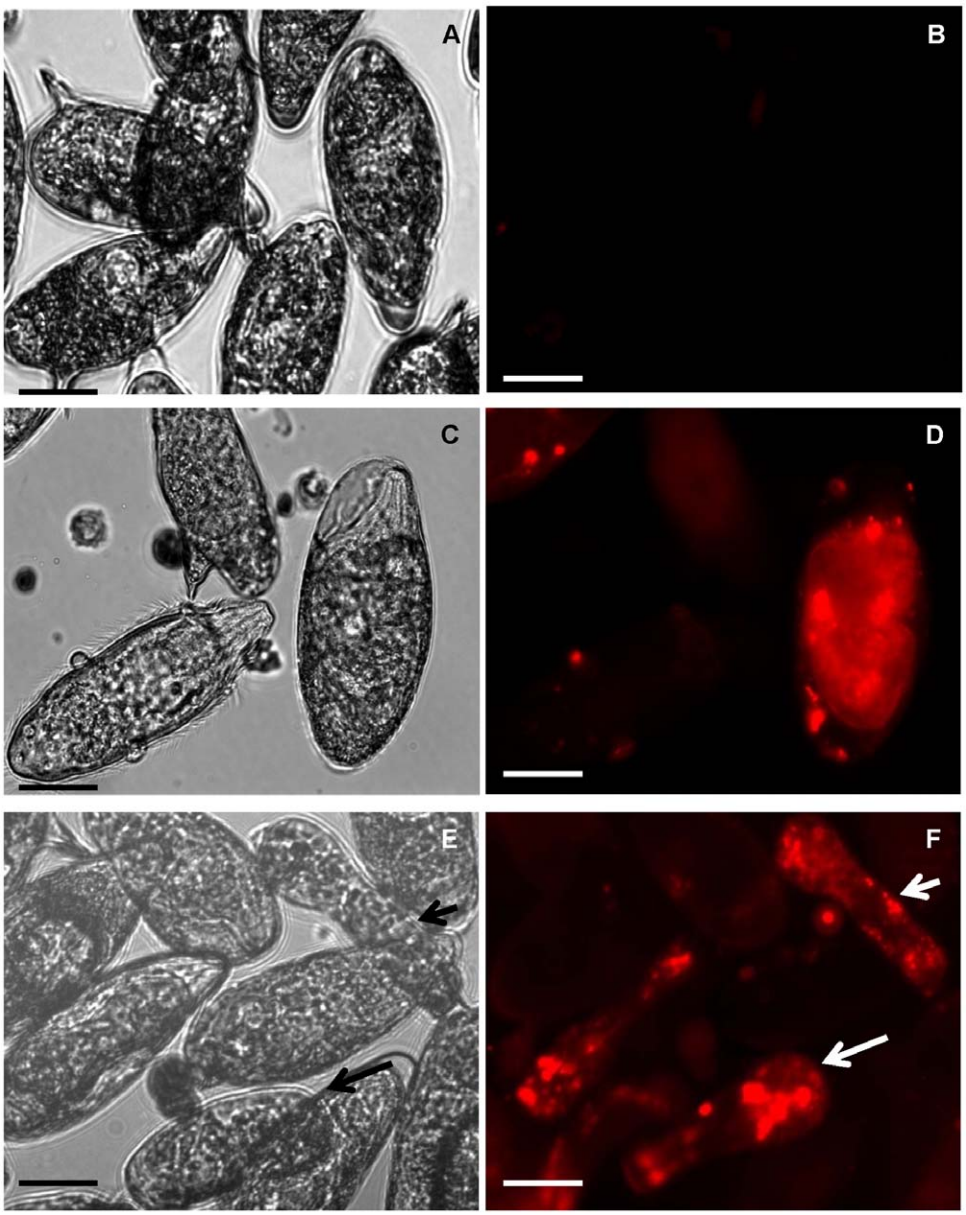

Figure 2. Fluorescent labeled short interfering RNA enters cultured eggs of *Schistosoma mansoni* after square wave electroporation. Representative images of schistosome eggs and miracidia 24 hours after electroporation with Cy3-siRNA; bright field, upper panels; fluorescence field, lower panels. Eggs were electroporated in medium containing 50 ng/μl of Cy3-siRNA. No Cy3-siRNA treatment control (A, B), Cy3-siRNA treated, fluorescent eggs (C, D) and fluorescent eggs and miracidium/sporocyst (arrows in E, F). Scale bar, 20μm.
doi:10.1371/journal.pntd.0000593.g002

copies were seen in the electroporated group compared to the soaked group (Figure 5A).

In order to estimate the ratio between the copy number of the transgene and that of a single-copy gene, we performed a relative quantification by qPCR. For every sample we performed real time PCR targeting luciferase and *Sm*CathD, a representative single copy gene. We saw a range of ratios among the four groups of gDNAs in both experiments 1 and 2, ranging from 0.03 copies of the FLuc transgenes for each copy of *Sm*CathD (no. 1, soaked) to 0.22 copies (no. 2, electroporated) (Figure 5B). More specifically, in each of the two experiments, the copy number of FLuc was 2 to 3 times higher in the electroporated group than in the soaked group. Together, these data indicate that square wave electroporation is more effective than soaking alone for delivering VSVG-MLV virions into eggs of *S. mansoni*.

Discussion

The schistosome egg represents an attractive developmental stage at which to target transgenes because it is readily obtained from experimentally-infected rodents or naturally infected people, is easily maintained in vitro, has a high ratio of germ to somatic cells and contains miracidia that can be employed to infect snails to propagate the life cycle. Furthermore, from the clinical perspective, the egg represents the major source of pathogenesis in human schistosomiasis. Here we observed that exogenous macromolecules penetrate into cultured eggs, and we speculate that small macromolecules such as Cy3-Silencer siRNA (13.8 kDa) readily enter eggs through the pores that anastomose throughout the eggshell and which provide access from sub-shell envelope and

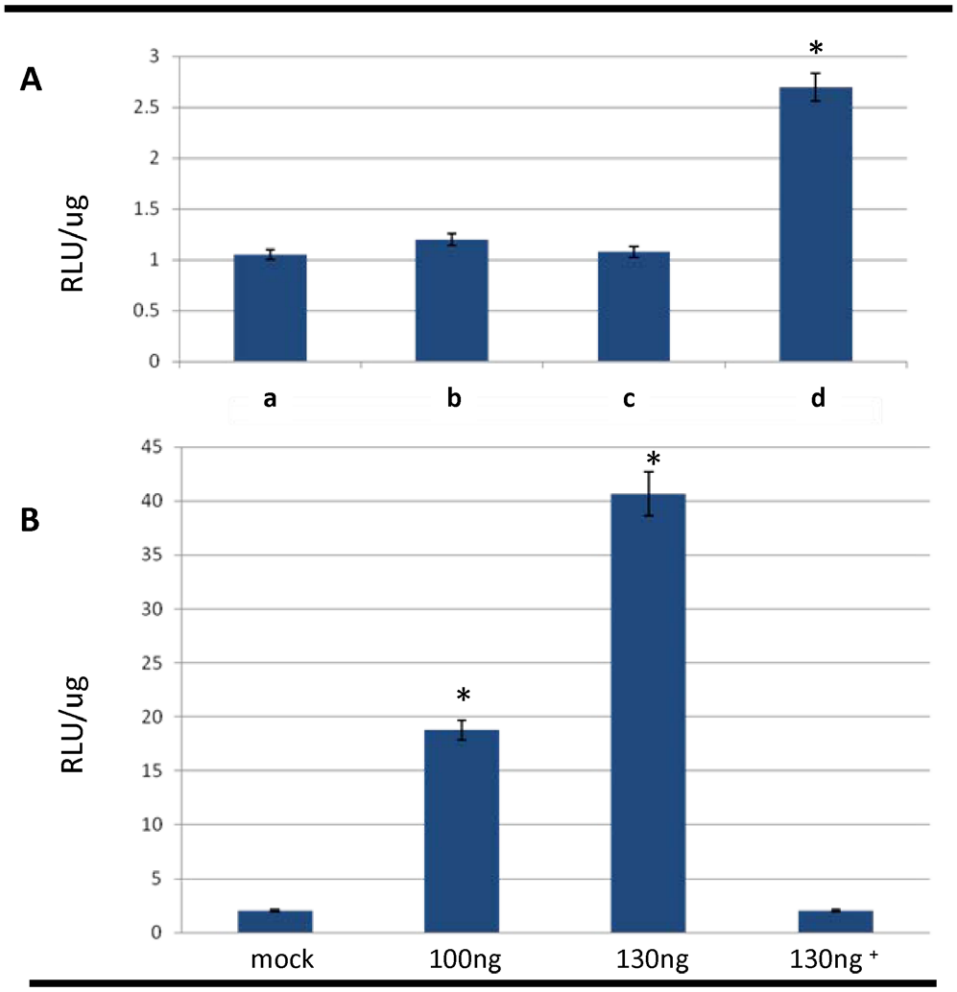

Figure 3. Luciferase activity in *Schistosoma mansoni* eggs. Detection of luciferase activity in extracts of eggs treated with capped mRNA encoding firefly luciferase (mFLuc). Panel A: luciferase activity three hours after soaking or electroporation with 130 ng/µl of mFLuc; a, negative controls soaked without mFLuc; b, eggs soaked with mFLuc; c, negative control eggs electroporated without mRNA; d, eggs electroporated with mFLuc. RLUs/µg, relative light units per microgram of egg protein. Panel B: luciferase activity three hours after electroporation of eggs in control (mock) and experimental groups with 100ng/µl and 130ng/µl of mFLuc, and at 30 hours after electroporation with 130 ng/µl (indicated with cross symbol). Asterisks denote statistically significant differences ($P \leq 0.05$) among groups.
doi:10.1371/journal.pntd.0000593.g003

the developing miracidium to the exterior [21,22]. Interestingly, after exposure to fluorescent siRNA, strong foci of fluorescence were distributed at the posterior of the larva, where the germinal cells are located [17]. This suggests that germinal cells can be reached by reporter transgenes introduced into schistosome eggs.

Luciferase activity was detected in extracts of eggs three hours after electroporation of capped mRNA, but not after soaking alone. This outcome may reflect the labile nature of the luciferase mRNA, with quick entry of the mRNA into eggs precipitated by electroporation allowing translation before mRNA degradation. At 512 kDa/1652 nt, mRNA encoding firefly luciferase is a far larger macromolecule than Cy3-siRNA. We also electroporated eggs in the presence of VSVG-MLV virions, a massive particle of $>10^8$ kDa [35]. Proviral MLV transgenes were detected in the miracidia and eggs using direct end-point PCR and qPCR.

The MLV virion is ~100 nm in diameter [36], whereas the diameter of the cribriform pores on the surface of the schistosome eggs is ~34 nm [22]. Thus it was remarkable that the virions apparently entered the eggs. In addition, beneath the eggshell there is an outer envelope, Reynold's layer, comprised of a fibrous matrix and a cellular inner envelope (von Lichtenberg's envelope) surrounding the developing miracidium [17,21,22,37]. Serpiginous branching channels from the eggshell pores traverse the eggshell allowing molecules to cross the eggshell barrier, as shown by the soaking of dsRNA [16,19]. Perhaps the electroporation causes an expansion of the diameter of the natural cribriform pores, or even establishes transient pores in the egg shell itself [38,39], through which the virions and mRNA can be propelled into the eggs. In single cell systems, reversible membrane breakdown accompanies electroporation, providing the pulse time is brief. Under these conditions, short-lived perturbations (electropores) can form in membranes, allowing transient access to the cytosol. The electropores reseal quickly at 37°C, but permit ingress of macromolecules and particles including hormones, proteins, RNA, DNA and organelles without deterioration of cellular functions [38,39]. Accordingly, electroporation may have produced electropores in the eggshell, the subshell envelope and/or cells of the developing miracidia through which the transgenes and/or virions entered cells of the schistosome larva. Even if electroporation ruptured or otherwise damaged the eggs, sufficient

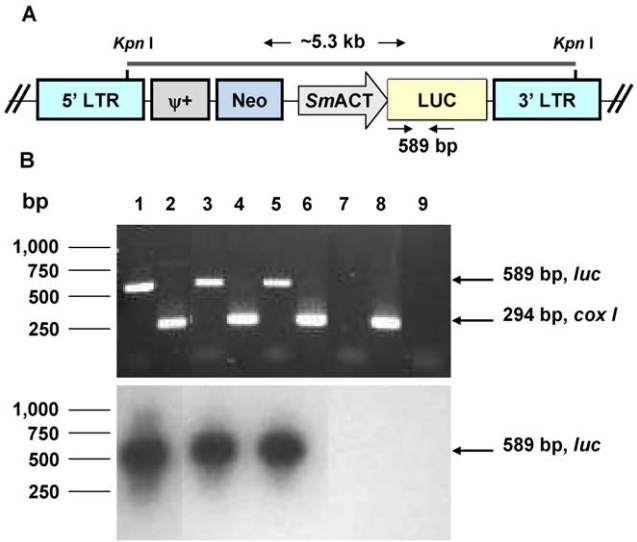

Figure 4. Detection by end-point PCR of retroviral transgenes in miracidia hatched from virion-transduced eggs. Panel A: Schematic representation of retroviral construct pLNHX-*Sm*ACT-Luc, showing position of *Kpn* I fragment employed as the hybridization probe. The retrovirus cassette included the firefly luciferase reporter gene (LUC) driven by the *S. mansoni* actin 1.1 gene promoter (*Sm*ACT), flanked by the 5′ and 3′ long terminal inverted repeats of the murine leukemia virus (5′LTR and 3′LTR). The cassette also included the gene endowing neomycin resistance (Neo) and the psi motif (ψ^+), involved in packaging the viral DNA). Panel B: Top panel: ethidium-stained PCR products resolved in agarose gel. Genomic DNAs (gDNA) from miracidia hatched from eggs transduced with pLNHX-*Sm*ACT-Luc virions were employed as templates for PCR using primers specific for *luc* transgene (lanes 1, 3, 5, 7) and *cox I*, a positive control endogenous schistosome gene, (lanes 2, 4, 6, 8). A reaction without template gDNA with primer pairs specific for the *luc* gene served as the negative control (lane 9). A gDNA sample (from transduced sporocysts) known to be positive for integrated transgenes was included as the positive PCR control for *luc* and *cox I* (lanes 1, 2). The miracidia analyzed in lanes 3 (*luc*) and 4 (*cox I*) were hatched from eggs electroporated in virus, and the miracidia analyzed in lanes 5 (*luc*) and 6 (*cox I*) were hatched from eggs soaked in virus. gDNA from non-transduced, control adults (negative control) were used as template for lanes 7 (*luc*) and 8 (*cox I*). Molecular size standards in base pairs (kb) are shown at the left, while the sizes of signals for *luc* (589 bp) and *cox 1* (294 bp) are indicated at the right. Bottom panel: autoradiograph of Southern hybridization signals from the PCR products (visualized in top panel) to a radiolabeled probe, a ~5.3 kb *Kpn* I fragment of pLNHX-*Sm*ACT-Luc spanning the genes encoding neomycin resistance (*neo*) and firefly luciferase (*luc*) (panel A).
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integrity may have been retained in many of them to allow the transformed miracidium to successfully hatch.

Quantitative real time PCR (qPCR) has been validated as a tool to ascertain transgene copy number and is as sensitive as Southern and dot blot hybridization [32,40,41]. We employed qPCR to estimate the copy number of the luciferase transgene and thereby evaluate the transduction efficiency of VSVG-MLV virions introduced into cultures of schistosome eggs by electroporation compared to soaking. The absolute quantification revealed the presence of 2–3 times more copies of the transgene in the electroporated compared to soaked eggs, indicating that electroporation was more efficient than soaking for transducing schistosome eggs. The outcome of the relative qPCR analysis was consistent with findings for absolute copy number of the transgene. Thus, since *S. mansoni* is diploid, somatic cells have two copies of each autosomal gene. Given that *Sm*CathD gene is a single copy gene [33], and that electroporation lead to the presence of ~20 copies of the transgene for every 100 copies of *Sm*CathD, i.e. a transgene copy number of 0.2, we speculate that 20 copies of the luciferase transgene were distributed in every 50 cells. However, we do not yet know how many copies of the transgene were present in any specific cell, genome or indeed egg.

Ascertainment of relative copy number of the transgene in comparison to the copy number of an endogenous gene would be informative and diagnostic in approaches for germ line transgenesis. A relative copy number of ≥1, comparing the transgene with an endogenous single copy gene, is expected for transgenic organisms where all the cells will include at least one copy of the transgene. By contrast, the copy number of ~0.2 we observed here reflects the situation that the transgene was not present in every cell of the transduced population of schistosome eggs. Indeed, we consider that most of the luciferase genes would have been located in cells at the periphery of the developing miracidium because these cells would be more likely to be transduced by the electroporated virions than cells deeper within the larva. (VSVG-MLV virions are replication deficient – after transduction of the cell, no virus is produced and so neighboring and/or deeper tissues remain uninfected.) In addition, since there is a high ratio of germ cells to somatic cells in the egg, and given that the location of the germ cells in the mature eggs has been established [17], it would be advantageous to introduce as many copies as possible of the transgene into this developmental stage in order to increase the likelihood of germ line integration.

These findings represent the first report of the utility of square wave electroporation for the introduction of exogenous macromolecules and virions into the schistosome egg. The egg/miracidia stages are attractive targets for transgenesis because they are rich in germ line cells. The transgenes may enter the eggs through the cribriform pores known to form networks from the exterior of the eggshell, and/or through electropores in as yet undetermined sites in the eggshell or surfaces of cells of the developing miracidium. In any event, these approaches confirm the egg stage as a tractable target for germ line transgenesis. They also are of potential use for investigating novel therapeutic interventions since eggs trapped in liver, and other organs, are the direct agents of pathogenesis in schistosomiasis.

Supporting Information

Figure S1 Representative low magnification images (5×) of *Schistosoma mansoni* eggs and miracidia in culture 24 hours after exposure to Cy3-siRNA. (A) Eggs in culture soaked in Cy3-siRNA, 50 ng/μl. Mock control without Cy3-siRNA (a, bright field; b, fluorescence field), and Cy3-siRNA treated eggs and miracidia (c, bright field; d, fluorescence field). (B) Eggs electroporated in the presence of 50 ng/μl of Cy3-siRNA. Mock control without Cy3-siRNA (a, bright field; b, fluorescence field), Cy3-siRNA treated eggs and miracidia (c, bright field; d, fluorescence field). Scale bar, 100 μm.
Found at: doi:10.1371/journal.pntd.0000593.s001 (0.60 MB PDF)

Figure S2 Representative high magnification images (40×) of *Schistosoma mansoni* eggs, miracidia and sporocysts in culture 24 hours after soaking with Cy3-siRNA are shown. (A, B) (Bright and dark fields, respectively) Representative images of two eggs, one of them exhibiting fluorescent spots within the larvae. (C, D) (Bright and dark fields, respectively) Representative images of an egg, miracidium and sporocyst. Arrowhead, ciliated plate shed from a miracidium. Spo, sporocyst, Mir, miracidium. Scale bar, 20 μm.

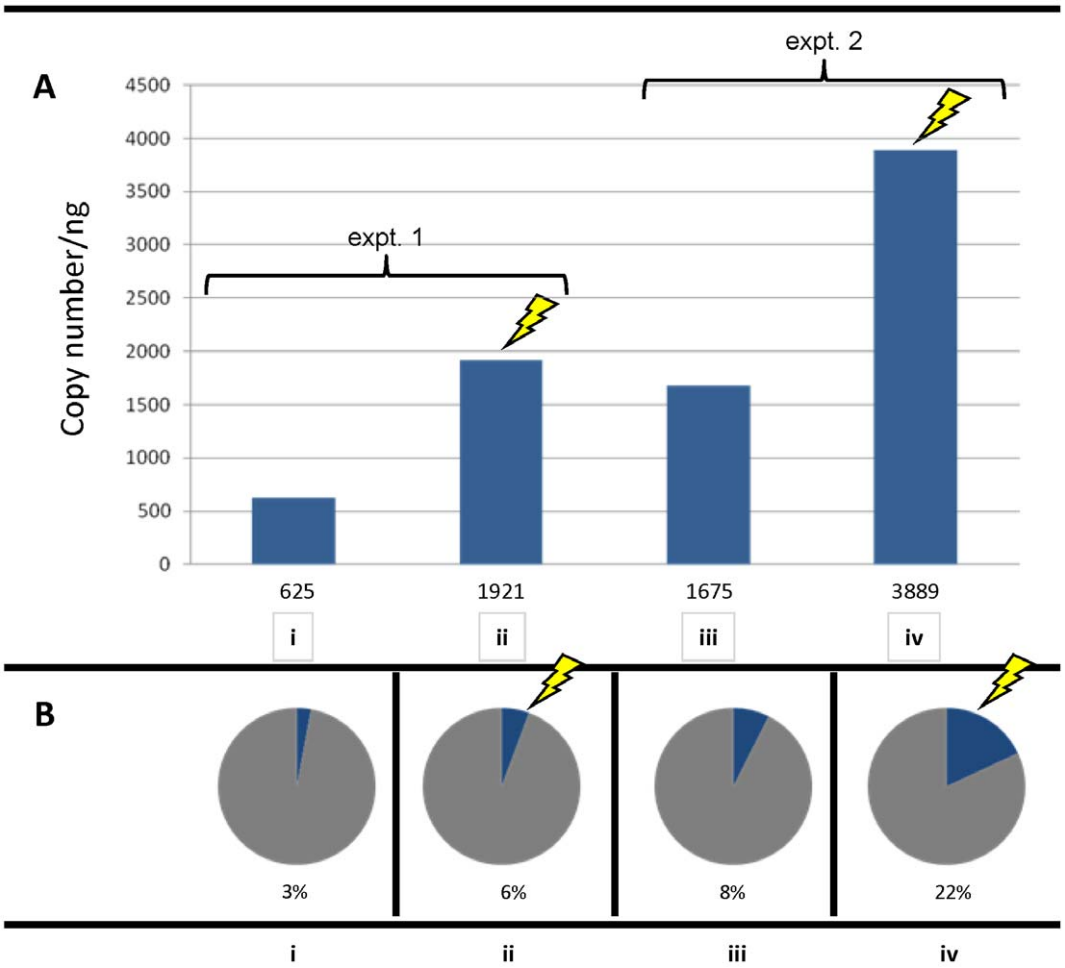

Figure 5. Copy numbers of luciferase transgenes ascertained by quantitative PCR. Panel A: Absolute copy number of the firefly luciferase (FLuc) transgene per ng of genomic DNAs from miracidia hatched from virion-exposed eggs - experiment (expt.) number 1 (i, soaking; ii, electroporation), experiment number 2 (iii, soaking; iv, electroporation). The absolute copy numbers are indicated below the bars. Panel B: Relative FLuc transgene copy number in comparison to the control *Sm*CathD (cathepsin D) single copy gene; the percentages represent the copy number of FLuc for every 100 copies of the cathepsin D gene. Transduced eggs from experiment no. 1 (i, soaking; ii, electroporation) and from experiment no. 2 (iii, soaking; iv, electroporation). The lightning flashes indicate treatment with electroporation.
doi:10.1371/journal.pntd.0000593.g005

Found at: doi:10.1371/journal.pntd.0000593.s002 (0.51 MB PDF)

Figure S3 Representative high magnification images (40×) of *Schistosoma mansoni* eggs, miracidia and sporocysts in culture 24 hours after electroporation with Cy3-siRNA are shown. (A, B) (Bright and dark field, respectively) Representative images of eggs, one of them with fluorescent spots within the larvae (white arrow). (C, D) (Bright and dark field, respectively) Images of an egg, miracidium and sporocyst. Arrowhead, ciliated plate shed from a miracidium. Spo, sporocyst, Mir, miracidium. Scale bar, 20 μm.
Found at: doi:10.1371/journal.pntd.0000593.s003 (0.47 MB PDF)

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Author Contributions

Conceived and designed the experiments: KJK GR JFT PJB. Performed the experiments: KJK GR TIO PJB. Analyzed the data: KJK GR MEM VHM JFT PJB. Contributed reagents/materials/analysis tools: KJK GR TIO MEM VHM PJB. Wrote the paper: KJK GR JFT PJB.

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B.3. Desarrollo de herramientas de perdida de función génica estable mediante la transgénesis con vectores codificantes de ARNs horquillas

ARTICULOS EN ANEXO I

- shRNAs expresados bajo el control del promotor del gen U6 de *Schistosoma mansoni* induce ARN de interferencia en células de fibrosarcoma humanas y schistosomulas

  Ayuk MA*, Suttiprapa S*, **Rinaldi G**, Mann VH, Lee CM, Brindley PJ
  (* Igual contribución al trabajo)

  International Journal for Parasitology 41 (2011), 783-789

- El promotor humano U6 promueve una mayor actividad de shRNA que su ortólogo de schistosoma en *Schistosoma mansoni* y células de fibrosarcoma humanas

  Raphaël Duvoisin *, Mary A. Ayuk *, **Gabriel Rinaldi**, Sutas Suttiprapa, Victoria H. Mann, Clarence M. Lee, Nicola Harris, Paul J. Brindley
  (*Igual contribución al trabajo)

  Transgenic Research (2011) doi:10.1007/s11248-011-9548-0

C. TRANSFERENCIA DE TECNOLOGÍA A OTROS PARÁSITOS TREMATODOS

C.1. Generación de herramientas de estudio de función génica en *Schistosoma haematobium*

ARTICULO: Manipulación genética de *Schistosoma haematobium,* el schistosoma desatendido

Gabriel Rinaldi, Tunika I. Okatcha, Anastas Popratiloff, Mary A. Ayuk, Sutas Suttiprapa, Victoria H. Mann, Yung-san Liang, Fred A. Lewis, Alex Loukas, Paul J. Brindley

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PLoS NEGLECTED TROPICAL DISEASES

Genetic Manipulation of *Schistosoma haematobium*, the Neglected Schistosome

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Abstract

Background: Minimal information on the genome and proteome of *Schistosoma haematobium* is available, in marked contrast to the situation with the other major species of human schistosomes for which draft genome sequences have been reported. Accordingly, little is known about functional genomics in *S. haematobium*, including the utility or not of RNA interference techniques that, if available, promise to guide development of new interventions for schistosomiasis haematobia.

Methods/Findings: Here we isolated and cultured developmental stages of *S. haematobium*, derived from experimentally infected hamsters. Targeting different developmental stages, we investigated the utility of soaking and/or square wave electroporation in order to transfect *S. haematobium* with nucleic acid reporters including Cy3-labeled small RNAs, messenger RNA encoding firefly luciferase, and short interfering RNAs (siRNAs). Three hours after incubation of *S. haematobium* eggs in 50 ng/μl Cy3-labeled siRNA, fluorescent foci were evident indicating that labeled siRNA had penetrated into miracidia developing within the egg shell. Firefly luciferase activity was detected three hours after square wave electroporation of the schistosome eggs and adult worms in 150 ng/μl of mRNA. RNA interference knockdown (silencing) of reporter luciferase activity was seen following the introduction of dsRNA specific for luciferase mRNA in eggs, schistosomules and mixed sex adults. Moreover, introduction of an endogenous gene-specific siRNA into adult schistosomes silenced transcription of tetraspanin 2 (*Sh-tsp-2*), the apparent orthologue of the *Schistosoma mansoni* gene *Sm-tsp-2* which encodes the surface localized structural and signaling protein Sm-TSP-2. Together, knockdown of reporter luciferase and *Sh-tsp-2* indicated the presence of an intact RNAi pathway in *S. haematobium*. Also, we employed laser scanning confocal microscopy to view the adult stages of *S. haematobium*.

Conclusions: These findings and approaches should facilitate analysis of gene function in *S. haematobium*, which in turn could facilitate the characterization of prospective intervention targets for this neglected tropical disease pathogen.

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

More people are infected with *Schistosoma haematobium* than with the other schistosomes combined. Of >110 million cases of *S. haematobium* infection in sub-Saharan Africa, 70 million are associated with hematuria, 18 million with major bladder wall pathology, and 10 million with hydronephrosis leading to severe kidney disease [1,2,3]. In many patients, chronic inflammation in response to *S. haematobium* ova leads to squamous cell carcinoma of the bladder [4,5]. *S. haematobium* is classified as a Group 1 carcinogen by the World Health Organization's International Agency for Research on Cancer [6,7] although the cellular and/or molecular mechanisms linking *S. haematobium* infection with cancer formation have yet to be defined [8]. One quarter to three quarters of women infected with *S. haematobium* suffer from female genital schistosomiasis (FGS) of the lower genital tract [1]. FGS results from deposition of the schistosome eggs in the uterus, cervix, vagina and/or vulva, with ensuing host inflammatory responses comprised of granulomas, fibrosis, and pathological localized blood vessel formation [9]. FGS increases susceptibility to HIV/AIDS [10,11,12], and decreases female fertility [13].

Given the enormous numbers of people infected with S. *haematobium*, and the pathogenesis of *S. haematobium* infection, including its association with bladder cancer and HIV/AIDS,

Author Summary

More people are infected with *Schistosoma haematobium* than other major human schistosomes yet it has been less studied because of difficulty in maintaining the life cycle in the laboratory. *S. haematobium* might be considered the 'neglected schistosome' since minimal information on the genome and proteome of *S. haematobium* is available, in marked contrast to the other major schistosomes. In this report we describe tools and protocols to investigate the genome and genetics of this neglected schistosome. We cultured developmental stages of *S. haematobium*, and investigated the utility of introducing gene probes into the parasites to silence two model genes. One of these, firefly luciferase, was a reporter gene whereas the second was a schistosome gene encoding a surface protein, termed *Sh-tsp-2*. We observed that both genes could be silenced – a phenomenon known as experimental RNA interference (RNAi). These findings indicated that the genome of *S. haematobium* will be amenable to genetic manipulation investigations designed to determine the function and importance of genes of this schistosome and to investigate for novel anti-parasite treatments.

there is a pressing need for new approaches to control including the development of a vaccine to prevent infection with *S. haematobium*. With regard to fundamental aspects of the host-parasite relationship, research on *S. haematobium* is in its infancy compared to *S. mansoni* and *S. japonicum* [14]. There have been massive recent advances in genomic, transcriptomic, and proteomic datasets for both *S. japonicum* and *S. mansoni* [15,16,17]. There now is an urgent need to establish similar datasets for *S. haematobium*, and in addition to establish tools and approaches to determine the function and importance of these schistosome genes - including *S. haematobium*-specific genes [14]. Here we cultured several developmental stages of *S. haematobium* and applied several functional genomics approaches to this species. We report that this schistosome, like *S. mansoni* and *S. japonicum*, is amenable to transformation with nucleic acid probes. Notably, the findings indicated the presence of an intact, active RNA interference pathway in *S. haematobium*, the neglected schistosome.

Materials and Methods

Schistosoma haematobium

Eggs of an Egyptian strain of *S. haematobium* were isolated from either small intestines, that had been thoroughly rinsed in 1× PBS to remove the gut contents, or livers of experimentally infected Syrian golden hamsters [18] following a protocol optimized for isolating eggs of *S. mansoni* from livers of mice [19]. In brief, three to five livers or two to three washed small intestines were chopped finely with a scalpel blade, and then blended to a smooth consistency in 50 ml of phosphate-buffered saline, pH 7.4 (PBS), 5 ml of 0.5% clostridial collagenase (Sigma) and 500 µl of polymyxin B (Sigma). Digests were incubated with gentle shaking at 37°C overnight, after which the contents were subjected to centrifugation at 400×*g* for 5 min. The supernatant was removed and the pellet resuspended in 50 ml PBS. This wash procedure was repeated twice more, with the exception that after the final centrifugation the pellet was resuspended into 25 ml of PBS. The resuspended mixture from liver was passed sequentially through 250 and 150 µm sieves. No passes through sieves were performed with the gut mixture. The liver mixture filtrate or the gut mixture were centrifuged at 400×*g* for 5 min, the supernatant discarded and the pellet resuspended in 3 ml of PBS. This was applied to a column of Percoll, prepared by mixing 8 ml of Percoll (GE Healthcare Bio-Science AB) with 32 ml of 0.25 M sucrose in a 50 ml tube. The tube was centrifuged at 800×*g* for 10 min. Liver or intestinal cells and debris that remained on the top of the Percoll were removed with a Pasteur pipette. The schistosome eggs, which pelleted tightly at the bottom of the tube, were washed three times with PBS and any residual host cells were removed by discarding the supernatant. Further purification of eggs was achieved by resuspension in 0.5 ml of PBS and application on to a second Percoll column, prepared by mixing 2.5 ml of Percoll with 7.5 ml of 0.25 M sucrose in a 15 ml polypropylene tube. The eggs were pelleted and then washed as before. Some eggs were snap frozen and stored at −80°C until use for extraction of total RNA. For other aliquots, the eggs were resuspended in 6 ml of complete culture medium - Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 100 U of penicillin and streptomycin (Invitrogen, Carlsbad, CA), split into 2 ml aliquots in a six-well plate and cultured at 37°C under 5% CO_2.

S. haematobium schistosomula were obtained by mechanical transformation of cercariae released from infected *Bulinus truncatus truncatus* snails and cultured at 37°C in modified Basch's medium under 5% CO_2 in air as described for *S. mansoni* schistosomula [20]. Mixed sex adults of *S. haematobium* were obtained by portal perfusion of infected hamsters followed by mesenteric vessel dissection and manual removal of adult worms using forceps under a magnification glass [18]. The adults were rinsed several times in PBS and cultured in complete culture medium.

Exposure of *S. haematobium* eggs to Cy3-siRNA

S. haematobium eggs were either electroporated and soaked in non-coding Cy3-labeled siRNAs (Silencer Cy3-Labeled Negative Control siRNA, Ambion, Austin, TX) at 50 ng/µl with conditions as described [21]. Briefly, eggs were washed in DMEM supplemented with 200 U/ml penicillin G sulfate, 200 mg/ml streptomycin sulfate, 500 ng/ml amphotericin B, 10 mM HEPES (wash medium) and transfected in 100 µl of the same medium in 4 mm gap cuvettes with an ElectroSquarePorator ECM830 (BTX, San Diego, CA) using a single square wave pulse of 125 volts of 20 milliseconds duration. After electroporation, eggs were washed in PBS three times to remove the unincorporated Cy3-labeled siRNA. Subsequently, eggs were transferred into complete DMEM at 37°C for three hours. Other eggs were soaked for three hours in Cy3-siRNA, then washed in PBS three times in order to remove the unincorporated Cy3-labeled siRNAs. The Cy3-siRNA exposed eggs, with or without electroporation, were examined under bright and fluorescent light (below) using a Zeiss Axio Observer A.1 inverted microscope fitted with a digital camera (AxioCam ICc3, Zeiss). Manipulation of digital images was undertaken with the AxioVision release 4.6.3 software (Zeiss). These manipulations were limited to insertion of scale bars, adjustments of brightness and contrast, cropping and the like; image enhancement algorithms were applied in linear fashion across the entire image and not to selected aspects.

Synthesis of mRNA, dsRNA, and siRNAs

To synthesize firefly luciferase mRNAs (mLuc), *in vitro* transcriptions of capped RNAs from PCR DNA templates were accomplished using the mMachine T7 Ultra kit (Ambion) as described [22,23]. Subsequently, RNAs were precipitated with ammonium acetate, dissolved in nuclease-free water and quantified by spectrophotometry (NanoDrop Technologies, Wilmington, DE). The dsRNAs were generated by *in vitro* transcription using, as templates, PCR products amplified with gene specific primers tailed

with the T7 promoter sequence. A luciferase dsRNA (dsLuc) template encoding the full length 1,672 kb was amplified from the pGL3-basic plasmid (Promega, Madison, WI), (F: 5′TAATACGACTCACTATAGGGTGCGCCCGCGAACGACATTTA-3′; R: 5′- TAATACGACTCACTATAGGGGCAACCGCTTCCCCGACTTCCTTA-3′). The siRNAs were designed with the assistance of the BLOCK-iT^{TM} RNAi Designer Tool, https://rnaidesigner.invitrogen.com/rnaiexpress/index.jsp. Block-iT^{TM} siRNA of 19 nt in length named si*Sh*TSP 2 (5′-GGA AUC CUG UUU CAA AGA U-3′), specific for residues 159–177 of the extracellular loop 2 of *S. haematobium* tetraspanin 2 (*Sh-tsp-2*) and an irrelevant siRNA (control) termed siScrambled, 5′-GGA GUC CCU UUA AAU AGA U-3′, the sequence of which included the same residues of si*Sh-tsp-2* but in which the order of the residues had been randomly mixed, were purchased from Invitrogen.

Transfection of developmental stages of *S. haematobium* with mRNA and/or dsRNA

S. haematobium eggs were maintained for one day after isolation from hamsters, then subjected to electroporation in the presence of mLuc at 150 ng/µl [21]. Briefly, ~2,000 eggs were subjected to the square wave electroporation in 4 mm gap pathway cuvettes (BTX) in 100 µl wash medium, as above. A group electroporated in the absence of mLuc was included as a mock-treated control. Thereafter the eggs were kept in culture for 3 or 20 hours, harvested and stored at −80°C. For RNAi approaches, one group of eggs was incubated with 30 µg of dsLuc, and other two groups were incubated without dsLuc. After 10 min at 23°C, 15 µg of mLuc was added to eggs in wash medium, except to a mock control group, i.e. a group of eggs not treated with exogenous nucleic acids. The eggs were subjected to square wave electroporation (above), transferred to pre-warmed culture medium and harvested three hours later.

Schistosomula of *S. haematobium* were removed from culture three hours after cercarial transformation, washed and resuspended in 100 µl of wash medium containing 30 µg of dsLuc. Two other groups of schistosomules were incubated in the absence of dsLuc, in 4 mm gap cuvettes. After 10 min incubation at 23°C, 15 µg of mLuc was added to the wash medium in each group, except to a mock control group. Thereafter the schistosomules were subjected to square wave electroporation, 125 V, 20 ms, transferred to prewarmed Basch's medium and harvested three hours later.

We have recently determined that dicing adult schistosomes into several fragments results in more reporter gene activity than in similar numbers of intact worms [24]. Accordingly, ~50 mixed sex adults of *S. haematobium* were removed from culture 24 hours after perfusion from hamsters, washed, diced into three or four fragments using a sterile blade. Intact or fragmented *S. haematobium* worms were placed into 4 mm gap pathway cuvettes in the presence of 15 µg of mLuc resuspended in 100 µl of wash medium and subjected to square wave electroporation, 125 V, 20 ms, one pulse. After electroporation, the worms and fragments were transferred into pre-warmed complete culture medium, incubated at 37°C under 5% CO_2 in air, and harvested 3 hours later.

For RNAi approaches targeting the luciferase reporter gene, the worms were diced into three or four fragments using a sterile blade, washed three times in wash medium and transferred to 4 mm gap cuvettes containing 100 µl wash medium. One group of diced adult worms was incubated with 30 µg of dsLuc, and the other two were incubated in the absence of dsLuc. Following incubation at 23°C for 10 min, 15 µg of mLuc was added to each group, except to the mock control group after which the parasites were subjected to a single pulse of square wave electroporation, 125 V, 20 ms. Subsequently, the diced worms were transferred to complete medium and maintained in culture; the worm fragments remained active (displaying movements) during the study.

For RNAi targeting an endogenous *S. haematobium* gene, intact adult worms were electroporated in the presence of 10 µg of si*Sh-tsp-2* or 10 µg of siScrambled in 100 µl (16.5 µM) of wash medium. We targeted intact worms for this experiment, dealing with silencing of an endogenous gene, with the aim of determining whether a gross phenotype might accompany gene knockdown. After electroporation, worms were transferred to complete medium for 48 h, then stored at −80°C.

Luciferase activity

Developmental stages of *S. haematobium* were harvested three hours after electroporation unless otherwise indicated, washed three times with wash medium and stored as wet pellets at −80°C. Luciferase activity in extracts of parasites was determined using Promega's luciferase assay reagent system and a tube luminometer (Sirius, Berthold, Pforzheim, Germany) [22]. In brief, pellets of parasites were subjected to sonication (3×5 s bursts for schistosomula and adults and 5×5 s bursts for eggs, output cycle 4, Misonix Sonicator 3000, Newtown, CT) in 300 µl 1× CCLR lysis buffer (Promega). The sonicate was clarified by centrifugation at 20,800 *g*, 15 min, 4°C and the supernatant, containing the soluble fraction, analyzed for luciferase activity. Aliquots of 100 µl of soluble fraction were injected into 100 µl luciferin at 23°C, mixed, and relative light units (RLUs) determined 10 s later by the luminometer. Replicate samples were measured, with results presented as the average of the readings per mg of protein. The protein concentration in the soluble fraction of the schistosome extract was determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Recombinant luciferase (Promega) was included as a positive control.

Gene expression analysis of *Sh-tsp-2*

Expression of *Sh-tsp-2* mRNA was analyzed in adults of *S. haematobium* harvested 48 hours after RNAi treatment. Total RNA was extracted from the worms using the RNAqueus®-4PCR Kit (Ambion). Any residual DNA remaining in the RNA was removed by DNase digestion using TurboDNase (Ambion) and cDNA was synthesized from 100 ng of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Primers and TaqMan probes were designed with the assistance of Beacon Designer (Premier Biosoft International, Palo Alto, CA) to obtain probes targeting *Sh-tsp-2* and *S. haematobium* tropomyosin (*Sh*Trop) (GenBank L76202.1) genes, as follows: for *Sh*TSP 2, forward primer: 5′-GAT GCA TTA AGA GAA TTC GTA A- 3′; reverse primer: 5′-TGG TGG AGT GAC ATA ATC-3′; probe: 5′-/56-FAM/TGA AGA ATC AGC ACC ACA GCA TTG/3IABlk_FQ/-3′; for *Sh*Trop, forward primer: 5′-ATC CGA GAT TTA ACA GAA C-3′; reverse primer: 5′-CGC TAA GAG CTT TGT ATC-3′; probe: 5′-/56-FAM/TTC TCA GCC AGT AAG TCA TCT TCC AA/3IABlk_FQ/-3′.Quantitative PCRs were performed in triplicate, using 96-well plates (Bio-Rad), with an initial denaturation step at 95°C for 3 minutes followed by 40 cycles of 30 sec at 95°C and 30 sec at 50°C, using a thermal cycler (iCycler, Bio-Rad) and a Bio-Rad iQ5 detector to scan the plates in real time. Reactions were carried out in 20 µl volumes with primer-probe sets (*Sh-tsp-2*, *Sh*Trop) and Perfecta qPCR FastMix, UNG (Quanta Bioscience, Gaithersburg, MD). The relative quantification assay $2^{-\Delta\Delta Ct}$ method [25] was employed, using *Sh*Trop as the reference gene. Results were plotted as *Sh-tsp-2* gene expression level relative to the reference gene considering 1 = *Sh-tsp-2* relative expression level measured in the irrelevant control group.

Laser scanning confocal microscopical imaging

Adult flukes were fixed in 4% paraformaldehyde overnight, rinsed with PBS, then incubated in propidium iodine (PI) diluted 1:1000 for one day. The PI-stained worms were placed on polylysine coated 50 mm Petri dishes, covered with PBS, and examined using a Carl Zeiss LSM 710 confocal system. This system includes a Zeiss Axio Examiner Z1 upright microscope equipped with a 20×/1.0 water dipping objective lens, deployment of which seemed prudent for imaging entire schistosomes since this objective does not require a coverslip (which markedly diminishes spherical aberrations). Confocal images were captured using a Qasar 32-channel spectral detector. Briefly, the worms were simultaneously scanned with 488 and 561 nm laser lines (multiline argon and diode laser, respectively), while the backward light was registered in 1024×1024 lambda-stack images taken simultaneously at a spectral resolution of 9.6 nm. Thus, for each single optical section, 32 images were recorded covering the visible spectrum from 423–721 nm, allowing the analysis of each of the (1) reflected light, (2) autofluorescence, and (3) characteristic emission at 617 nm from PI. To detect the reflected laser light, we utilized a T80/R20 beamsplitter, which only partially attenuates the laser lines in the backward direction. Confocal stacks for three-dimensional (3D) rendering were taken at z-scaling of 1.7 µm, which matched the pinhole opening (34 µm). Pixel resolution was 0.59 µm. After completion of the online acquisition, a linear spectral unmixing protocol was applied to the lambda-stacks to generate two three-channel confocal stacks. To generate reliable spectral unmixed channels, various sites from the worm were tested and representative for the 488-line reflection, autofluorescence and PI were selected and used as reference for unmixing. Thus, the resulting images, encoded in three channels reflected light, autofluorescence and PI signals from the nuclei. Unmixed, confocal stacks were imported to Volocity (v.5.5, Perkin Elmer/Improvision) for further three-dimensional rendering and analysis.

Ethics statement

Male LVG hamsters were purchased from Charles River (Wilmington, MA) and maintained in the Biomedical Research Institute's (BRI) animal facility, which is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC; #000779), is a USDA registered animal facility (51-R-0050), and has an Animal Welfare Assurance on file with the National Institutes of Health, Office of Laboratory Animal Welfare (OLAW), A3080-01. Maintenance of the hamsters, exposure to *S. haematobium* cercariae, and subsequent harvesting of tissues were approved by the BRI Institutional Animal Care and Use Committee (protocol approval number 09-03). All procedures employed were consistent with the Guide for the Care and Use of Laboratory Animals.

Results

Culture of developmental stages of *Schistosoma haematobium*

Given the scarcity of reports on *in vitro* culture techniques focused on *S. haematobium* we adapted protocols from studies with *S. mansoni* [20], to maintain some developmental stages in culture. Thus, eggs isolated either from small intestines or livers of hamsters, schistosomula mechanically transformed from cercariae released by experimentally infected *B. t. truncatus* snails, and mixed sex adults from portal perfusion and mesenteric vessel dissection of hamsters were cultured in the indicated medium at 37°C, 5% CO_2. No differences in gross appearance were evident between the eggs isolated from intestines (Figure 1A and B) or liver (Figure 1C and D). The eggs were cultured in complete medium (Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 100 U of penicillin and streptomycin (Invitrogen, Carlsbad, CA), for up to seven days (not shown). Mixed sex adults were cultured in complete medium (above) for up to five days (Figure 2A). Notably, at higher magnification, longitudinal orientation of the eggs within the uterus of the female schistosome was apparent (Figure 2B) which is in marked contrast to the transverse disposition in utero of *S. mansoni* eggs, e.g. [26,27]. Schistosomula of *S. haematobium*, obtained by cercarial transformation as described above, were cultured in modified Basch's medium (Figure 2C and 2D).

Cy3-siRNA is effectively incorporated into eggs of *S. haematobium*

To investigate whether macromolecules could be introduced into *S. haematobium* eggs, cultures of eggs were incubated in a Cy3-siRNA (13.8 kDa) with or without concomitant square wave electroporation. Three hours after exposure to Cy3-siRNA, eggs were examined by fluorescence microscopy. Surprisingly, strong fluorescence including foci of intense fluorescence was revealed in Cy3-siRNA soaked eggs in contrast to those subjected to electroporation (Figure 3 and S1). More than 80% of the treated eggs emitted fluorescence as revealed at low magnification (Figure S1). These results indicated that it is possible to introduce Cy3-siRNA into *S. haematobium* eggs by simple soaking and that electroporation was not essential for this reporter probe. (However, with some stages, electroporation is more efficient: it mobilizes dsRNA or mRNA into the target worms quickly, which is advantageous when working with RNAs that are labile.) Although the structure of the *S. haematobium* eggshell is not well described, pores are present in the eggshell of *S. mansoni* eggs – the eggshell has been described as cribriform [28].

Reporter firefly luciferase is active in *S. haematobium*

To ascertain if transgene mRNAs could penetrate schistosome eggs and be translated into an active protein, we electroporated cultured eggs in the presence of firefly luciferase mRNA (mLuc) (512 kDa). More specifically, 48 hours after isolation 1,500–2,500 eggs were subjected to electroporation in the presence of 150 ng/µl of mLuc, and collected three and 20 hours later. Luciferase activity was detected in the mLuc electroporated group compared with untreated control at 3 h, and even higher luciferase activity was measured in eggs harvested at 20 h after electroporation (Figure 4A). (A signal of ~100–150 RLUs/sec/mg was measured in the mock control group, which represents the background baseline of this assay.) We electroporated intact and fragmented adult worms in the presence of 15 µg of luciferase mRNA, and measured the luciferase activity 3 hours later. Several fold (~3.5 times) more activity was detected in fragmented than intact worms (Figure 4B), in like fashion to *S. mansoni* [24]. Collectively, these findings indicated that square wave electroporation efficiently delivered exogenous nucleic acids into the eggs and adults of *S. haematobium* and that reporter luciferase was functionally translated from this exogenous mRNA.

dsRNA silences reporter luciferase mRNA in *S. haematobium*

We have reported that it is feasible to knock down an exogenous reporter transgene by dsRNA in order to detect an active RNAi pathway in flukes [23,29]. Given that *S. haematobium* can be productively transformed with mRNA by square wave electroporation, we proceeded to investigate silencing of expression of the

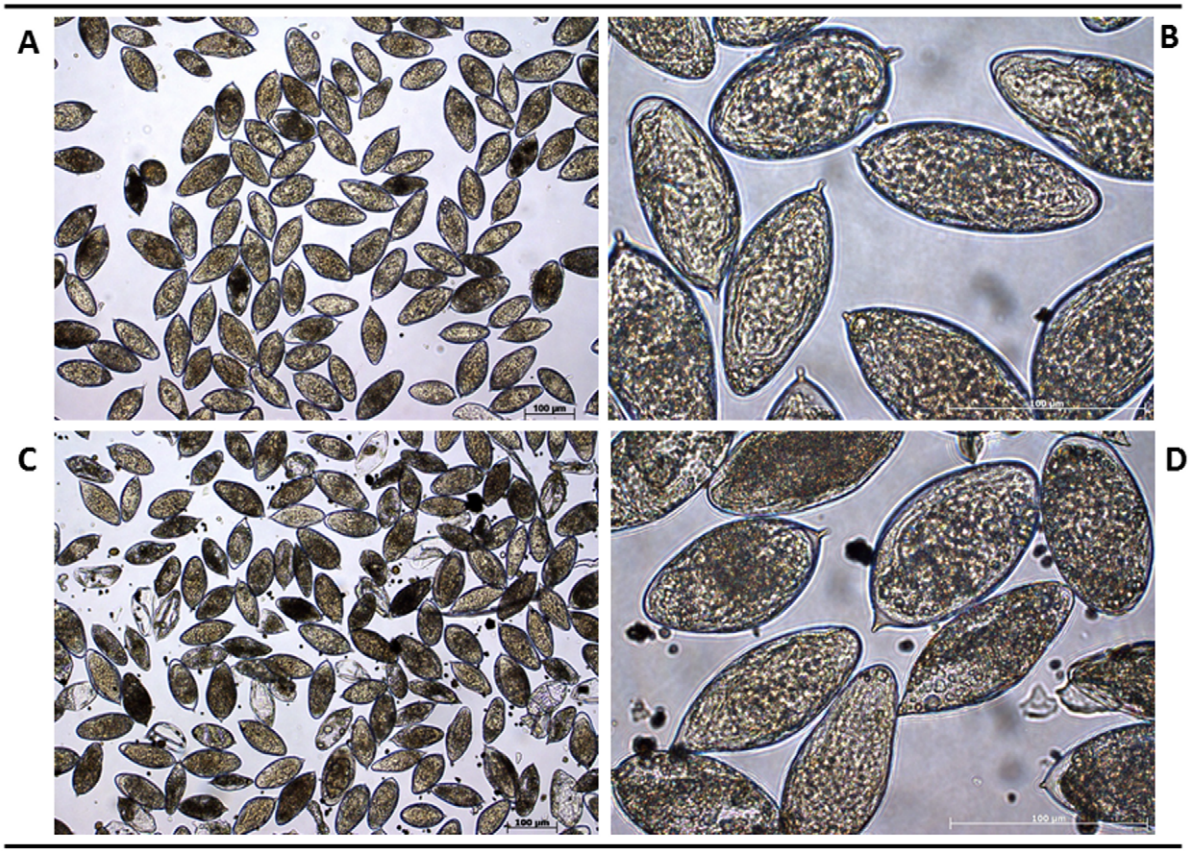

Figure 1. Eggs of *Schistosoma haematobium*. These schistosome eggs were obtained from experimentally infected hamsters, and thereafter were maintained in culture. Eggs were recovered from small intestines (panels A and B) or liver (panels C and D). Scale bars, 100 μm.
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exogenous reporter transcript (mLuc). About 2,000 eggs were removed from culture four days after isolation, washed and subjected to electroporation in the presence of both mLuc and dsLuc (mLuc+dsLuc group). Control eggs electroporated in the absence of exogenous RNAs (mock control) and positive control eggs electroporated in the presence of mLuc (mLuc group) were included (Figure 5A). Reduced luciferase activity was evident in the mLu*c*+dsLuc group in comparison with the mLuc group, even though the luciferase activity in terms of absolute RLUs/sec/mg measured in eggs at three hours after electroporation was relatively low in comparison to the other developmental stages (Figure 5B, left panel). (It appears to be more difficult to introduce mRNA into eggs than other developmental stages, likely because of the presence of the eggshell.) The experiment with eggs was repeated three times; knock down was apparent in two of the three trials. Fragmented adults were also examined; >75% knockdown of luciferase was observed (Figure 5B, center panel). The experiment was repeated; knock down was obvious on each occasion.

Furthermore, three hour old schistosomula were co-transfected with messenger RNA encoding luciferase (mLuc) and dsRNA targeting the luciferase transcript (dsLuc) by electroporation (mLu*c*+dsLuc group), along with controls (experimental design shown in Figure 5A). At 3 h after electroporation, luciferase activity of 14,080 RLU/sec/mg was evident in lysates of the positive control schistosomules transfected with mLuc. By contrast, luciferase activity in the schistosomules exposed to both dsLuc and mLuc was significantly lower, 6073 RLUs/sec/mg, representing 43% of the positive mLuc control (Figure 5B, right panel). In review, a similar trend was apparent in each of these developmental stages: it is feasible to knock down the reporter luciferase gene in eggs, schistosomules and adults of *S. haematobium*.

Suppression of an endogenous gene in adults of *S. haematobium*

In addition to reporter luciferase, we introduced siRNA specific for *Sh-tsp-2*, an orthologue of a *S. mansoni* membrane protein critical for tegument formation, the tetraspanin *Sm*-TSP-2 [30] into intact *S. haematobium* mixed sex adults. At 48 hours after electroporation of siRNAs – si*Sh-tsp-2* and a control siRNA, we observed a significant knock-down of levels of the *Sh-tsp-2* transcript (Figure 6). This experiment was repeated three times; knock down was seen on each occasion, and on two of these three occasions the knock-down was >75%. Notably, no gross phenotypic differences among the adult worms were evident by light microscopy (not shown).

Confocal micrographs highlight characteristic morphology of *S. haematobium*

In addition to the images of cultured stages of *S. haematobium*, adult worms were fixed in 4% paraformaldehyde and stained with PI. Spectral confocal microscopy was used to image the entire volume of the paraformaldehyde fixed male and female worms at high resolution (Figure 7A). We employed A T80/R20 beamsplitter to image the flukes, using backward scattered laser light. The reflected light is registered on the lambda stack as a dual-peak at the wavelength of the laser used for excitation. In this case, the

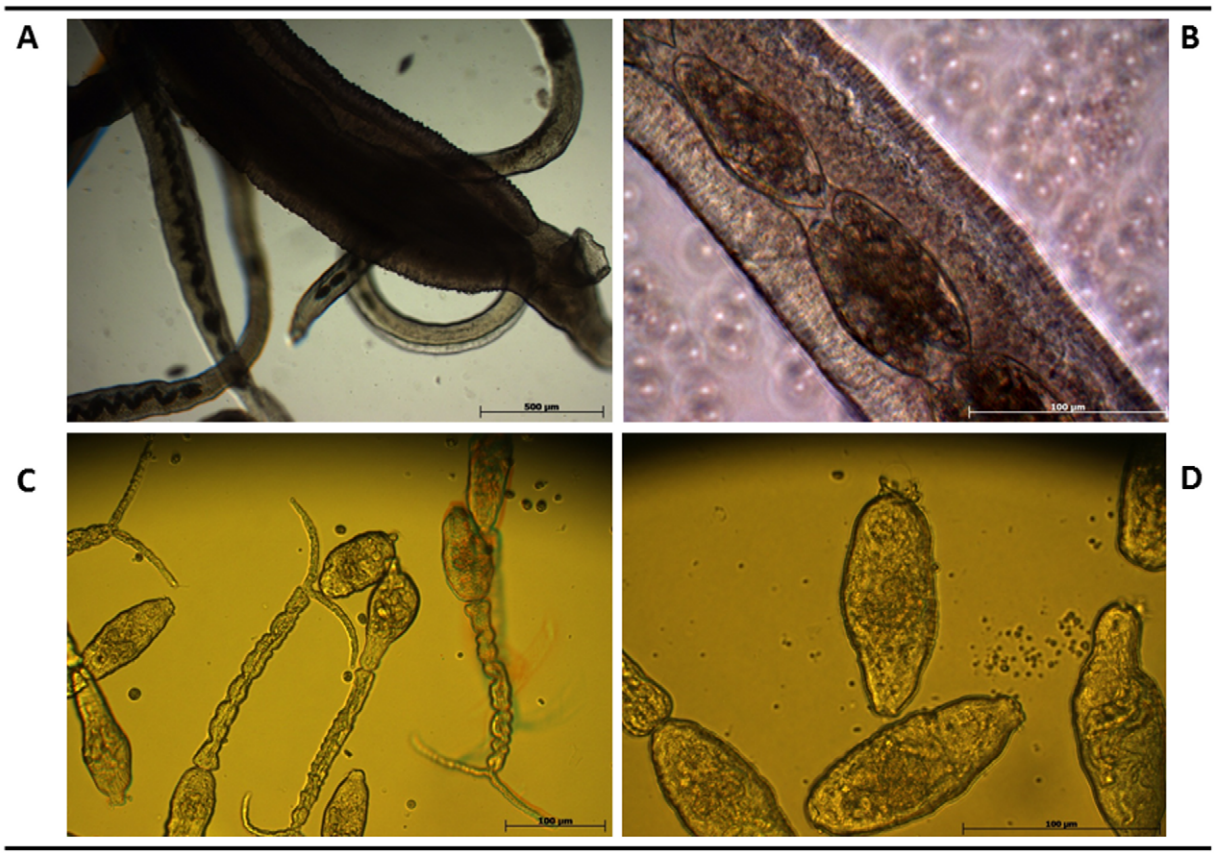

Figure 2. Adults and micrograph showing the characteristic longitudinal disposition of the eggs along the body of the schistosomules of *Schistosoma haematobium*. Panel A: micrograph illustrating a population of mixed sex adults obtained by portal perfusion from infected hamsters and maintained in culture. Panel B: high magnification micrograph showing the characteristic longitudinal disposition of the eggs along the body of the female. Panel C: images of cercariae released from infected snails. Panel D: images of representative schistosomules in culture 3 hours after cercarial transformation. Scale bars, 500 µm (A) and 100 µm (B, C and D).
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488 nm laser line produced a large reflection response, from which images of the surface of the schistosomes were assembled (Figure 7B and F). The approach also recorded in consistent and reproducible manner, autofluorescence deriving from the gut and, dramatically, eggs *in utero* (Figure 7D and E). The autofluorescence registered on the lambda stack displayed a broad spectrum - peak ~560 nm, range 500–650 nm (overlapping with numerous widely employed dyes and fluorescent proteins). The signals from nuclei stained with PI (Figure 7C and G) registered as a spectral curve (peak 617 nm) that partially overlapped with the red-shifted slope of the autofluorescence. Thus, we could select discrete sites on worms representing reflected light, autofluorescence and PI fluorescence that served as references for linear unmixing [31]. The three-channel confocal stacks, derived after linear unmixing, comprised channels representing the reflection, autofluorescence and PI signal at high signal to noise ratio. Figures 7E and 7H show three-dimensional images assembled from the merged reflected light, autofluorescence and the PI fluorescence signals.

Discussion

Using *S. haematobium* eggs from livers and intestines of experimentally infected hamsters, adult worms perfused from the hamsters and cercariae from *B. t. truncatus* snails, and using similar approaches to those for *S. mansoni* [20], we were able to culture eggs, schistosomules, and adults of *S. haematobium* and to subject these developmental stages to genetic manipulation. We transformed eggs of *S. haematobium* with a small nucleic acid probe, Cy3-siRNA. Experience with the other two major schistosomes has revealed that the schistosome egg represents an attractive developmental stage at which to target transgenes because it is readily obtained from experimentally-infected rodents or naturally infected people, is easily maintained in vitro, has a high ratio of germ to somatic cells and contains a miracidium that can be employed to infect snails to propagate the life cycle [21,32,33]. Furthermore, from the clinical perspective, the egg represents the major source of pathogenesis in human schistosomiasis haematobia. We observed that exogenous macromolecules penetrate into cultured eggs, and we speculate that small macromolecules such as Cy3-Silencer siRNA (13.8 kDa) enter eggs through the pores that likely anastomose throughout the eggshell and which provide access from sub-shell envelope and the developing miracidium to the exterior, in like fashion to the egg of *S. mansoni* [29,34,35].

Others and we have described the utility of firefly luciferase as a transgene probe in *S. mansoni* and the liver fluke *Fasciola hepatica* [21,22,23,29,36]. We have also reported the utility of luciferase as a model target to identify the presence of an active RNA interference pathway in less well studied helminth parasites, especially where genome sequences are unavailable [23]. Using this strategy, we now present findings that indicate for the first time the presence of an intact RNAi pathway in *S. haematobium*. In each of three developmental stages investigated – eggs, schistosomula, and mixed sex adults, co-introduction of dsRNA spanning

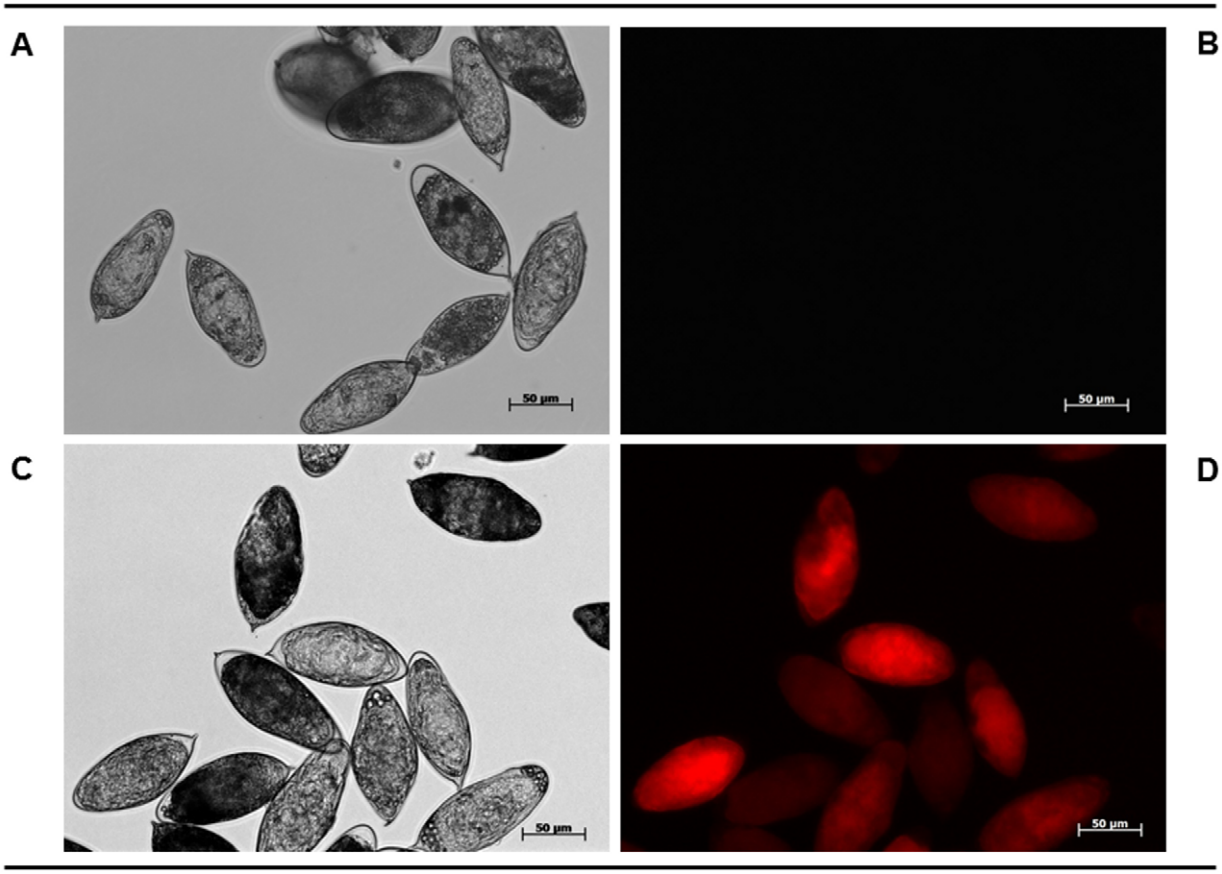

Figure 3. Labeled short interfering RNA enters cultured eggs of *Schistosoma haematobium*. Representative images of schistosome eggs 3 hours after soaking in Cy3-siRNA; panel A: no Cy3-siRNA treatment control, bright field; panel B: no Cy3-siRNA treatment control, fluorescence field, panel C: soaked eggs in medium containing 50 ng/µl of Cy3-siRNA, bright field, panel D: soaked eggs in medium containing 50 ng/µl of Cy3-siRNA, fluorescence field. Scale bar, 50 µm.
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the transcript of firefly luciferase and of mRNA encoding firefly luciferase resulted in robust knockdown of the exogenous mRNA. Luminometric measurement of luciferase activity provided a direct demonstration of gene silencing at the protein level.

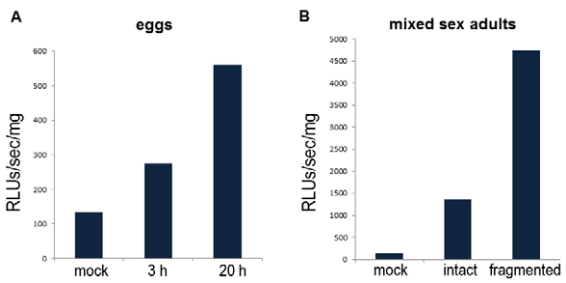

Figure 4. Luciferase activity measured in *Schistosoma haematobium*. Panel A: *S. haematobium* eggs transfected with 150 ng/µl of firefly luciferase mRNA. Detection of luciferase activity in mock control (mock) and in mLuc treated eggs, measured three (3 h) and 20 (20 h) hours after electroporation. Panel B: Luciferase activity measured in extracts of adult worms 3 h after electroporation, (mock) adult worms treated with no molecule, (intact) intact worms treated with 150 ng/µl mRNA, and (fragmented) worms diced into three or more pieces and treated with 150 ng/µl mRNA.
doi:10.1371/journal.pntd.0001348.g004

In *S. mansoni*, comparative studies indicate that efficiency of RNAi efficiency following electroporation is superior to passive soaking [37]. Here we employed square wave electroporation to introduce the dsRNA and luciferase mRNA into developmental stages of *S. haematobium*. Eggs, schistosomula and adults of *S. haematobium* were amenable to transfection with foreign nucleic acids using this technique. Given that these stages tolerated the electro-transfection conditions well, we anticipate that this technique can be optimized for genetic analysis and genomic manipulation of *S. haematobium*. Whereas soaking performed better than electroporation alone for eggs of *S. haematobium*, it will be worthwhile to employ electroporation followed by soaking of the transfected eggs, a combination that is superior to soaking alone in eggs of *S. mansoni* [21].

Although little is known about the protein encoding genes of *S. haematobium*, we obtained the sequence of *Sh-tsp-2*, an apparent orthologue of *Sm-tsp-2* which encodes a lead vaccine antigen for schistosomiasis mansoni [30]. By targeting the sequence encoding the extracellular loop 2 domain of this protein with a 19 nt siRNA, we observed strong knockdown of the *Sh-tsp-2* transcript in adult worms. Thorough studies targeting this gene are warranted given its performance as a vaccine antigen for *S. mansoni* infection and because of the integral role that *Sm*-TSP-2 plays in development, maturation or stability of the tegument [30].

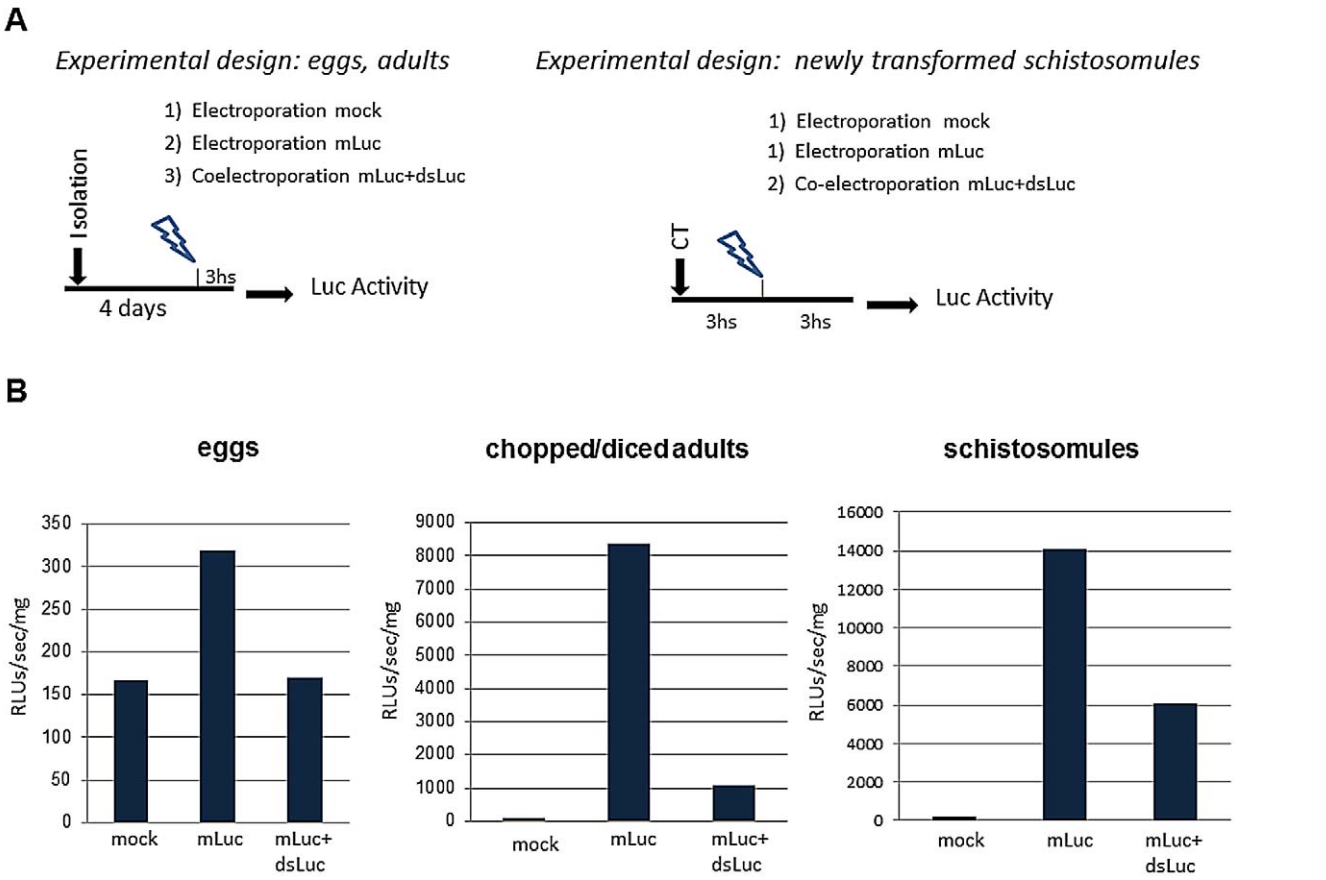

Figure 5. Suppression of exogenous luciferase activity in transfected eggs, chopped/diced adults and schistosomules of *Schistosoma haematobium*. Panel A: schematic representation of the experimental designs. Panel B: Luciferase activity measured in the indicated groups three hours after electroporation.
doi:10.1371/journal.pntd.0001348.g005

We deployed laser scanning confocal microscopy to view the adult stage of *S. haematobium*. In addition to facilitating views of the entire worms (≥1 cm in length), the approach circumvents barriers to reliable fluorescence imaging of schistosomes, including the notion of *autofluorescence* of schistosome eggs, e.g. [38]. The provenance of signals from the eggs (and gut) aside, the phenomenon deserves deeper exploration since it has the potential to chart predictable anatomical landmarks of the schistosome that can facilitate microanalysis of schistosome organs and tissues. Future characterization of native fluorescence signals of schistosomes can be expected to be of interest. Unlike fixed worms, evaluation of the living worms critically depends on minimizing invasive approaches. Also, fluorescence labels suitable for living cells generally cause some perturbation of normal functions. Thus, a library of spectrally distinctive signals – including the signal from eggs reported here - can be expected to facilitate microscopic imaging of viable schistosomes. Collectively, spectral confocal imaging provided the technological capacity to document eggs in utero of this neglected schistosome by extracting their emission of autofluorescence. Also, we imaged adult *S. haematobium* worms by staining tegumental nuclei with propidium iodide, which allowed the assembly of the three dimensional structure of the blood fluke. The spectral confocal microscopy approaches allowed differentiation of a fluorochrome from natural signals, e.g. PI versus autofluorescence, and portends its likely utility for monitoring reporter genes such as green fluorescent protein in transgenic schistosomes.

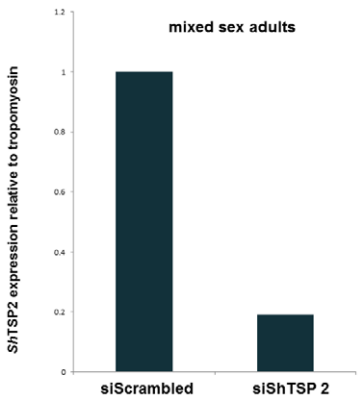

Figure 6. Silencing of the gene encoding the tetraspanin 2 antigen of *Schistosoma haematobium*. Quantitative RT-PCR analysis of the mRNAs from adult *S. haematobium* worms at 48 h after transfection by electroporation with siRNA specific for *Sh-tsp-2*. >80% silencing of the *Sh-tsp-2* (siShTSP2) evident when compared to the control group treated with siRNA scrambled control (siScrambled). *Sh-tsp-2* expression was normalized to a control mRNA encoding tropomyosin.
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In conclusion, this is the first report of genetic manipulation of *S. haematobium*. The procedures described here are expected to find application in determining the importance of *S. haematobium* genes.

Figure 7. Representative three dimensional (3D) renderings from laser scanning confocal images of adult forms of *Schistosoma haematobium*. Panel **A**: 3D rendering from the female (left) and male (right) *S. haematobium*. Images were captured with 5× objective as tile-scan to cover the entire worm in addition to z-stacks. Propidium iodide (PI) was used to label the nuclei (red). **B–E**: High power (20×/1.0) 3D rendering from a female *S. haematobium* capturing the anterior of the worm. Three channels were extracted after applying a linear spectral unmixing algorithm to a lambda stack confocal images. **B**, The surface of the female *S. haematobium* visualized using reflected light scattering from the 488 nm laser line. **C**, PI –labeling, **D**, 'autofluorescence' from the schistosome eggs, **E**, merge of **B–D**, showing the structure of the anterior of the worm, with a semi-transparent visualization. In **D** and **E**, arrows indicate location of schistosome eggs. **F–H**, 3D rendering from the anterior of a male worm visualized with a similar approach used in **B–E**. **F**, reflected light channel, **G**, PI channel, **H**, merge of **F** and **G** using semi-transparent visualization. Scale bars, 500 µm (**A**), 400 µm (**B–E**), 200 µm (**F–H**).
doi:10.1371/journal.pntd.0001348.g007

Whereas few sequences are yet available, there is now increasing interest in sequencing the *S. haematobium* genome. Tools and procedures for genetic and genomic manipulation of *S. haematobium* will soon be needed to determine the importance of prospective new gene targets for development of novel interventions.

Supporting Information

Figure S1 Representative micrographs at low magnification (4×) of *Schistosoma haematobium* eggs at three hours after exposure to Cy3-siRNA. Panels A and B: control without Cy3-siRNA, bright (A) and fluorescence (B) fields; panels C and D: soaked eggs in medium containing 50 ng/µl of Cy3-siRNA, bright (C) and fluorescence (D) fields. Panels E and F: control electroporated eggs without Cy3-siRNA, bright (E) and fluorescence (F) fields, panels G and H: eggs electroporated in the presence of 50 ng/µl of Cy3-siRNA, bright (G) and fluorescence (H) fields. Scale bar, 200 µm.
(TIF)

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Author Contributions

Conceived and designed the experiments: GR SS AP VHM AL PJB. Performed the experiments: GR TIO AP MAA SS. Analyzed the data: GR AP SS VHM AL PJB. Contributed reagents/materials/analysis tools: GR AP SS AL VHM Y-sL FAL PJB. Wrote the paper: GR AP FAL AL PJB. Confocal microscopy and imaging: AP SS GR.

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C.2. Desarrollo de la transgénesis mediada por vectores retrovirales en *Fasciola hepatica*

Buscando transferir la transgénesis mediada por retrovirus, desarrollada y optimizada en *Schistosoma mansoni,* a *Fasciola hepatica,* procedimos a transducir juveniles en cultivo con el virus de la leucemia murina modificado (MLV). Dos días luego de la transfección los juveniles fueron separados en dos grupos. Un grupo de parásitos fue fijado en paraformaldehído y procesado para inmunohistoquímica y así detectar proteínas virales en la superficie del organismo (**Figura 13 A**). Otro grupo de parásitos transducidos fue procesado para extracción de ADN genómico y su análisis buscando la presencia de genes reporteros codificados en el genoma viral. El PCR convencional, confirmado por hibridación Southern de los productos de amplificación, detectó la presencia de los reporteros GFP y *neoR* en el ADN proveniente de juveniles transducidos (**Figura 13 B**). Por PCR cuantitativo se estimó el número de copias de transgenes (gen de la luciferasa) en juveniles de *F. hepatica* transducidos con MLV ya sea mediante electroporación como la simple incubación de los parásitos en presencia de las partículas virales en el medio de cultivo (**Figura 13 C**).

Estos resultados preliminares, que deberán ser confirmados en el futuro, revelan un camino promisorio hacia la transgénsis mediada por vectores retrovirales en *Fasciola hepatica* y otros trematodos parásitos más desatendidos pero de gran importancia médica y veterinaria.

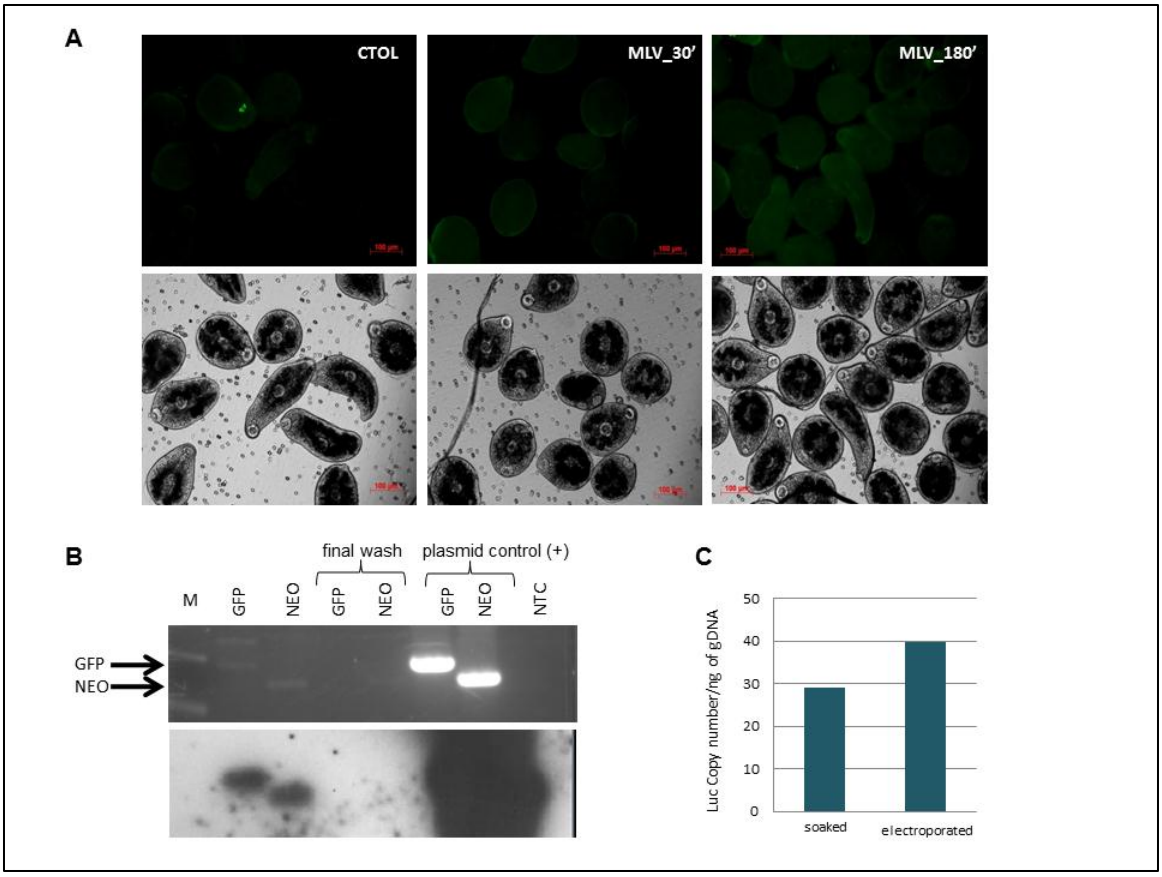

Figura 13. Transgénesis mediada por retrovirus en juveniles de Fasciola hepatica.
Panel A: Inmunolocalización de la proteína VSVG retroviral en la superficie de juveniles de F. hepatica, incubados en ausencia del retrovirus MLV (izquierda), incubados por 30 minutos (centro) o por 180 minutos (derecha) en presencia de MLV.
Panel B: Identificación de los transgenes retrovirales en el genoma de juveniles de F. hepatica transducidos con VSVG-Mo-MLV, mediante PCR directo contra ADN genómico. <u>Panel superior</u>: Productos de PCR resueltos en gel de agarosa teñidos con bromuro de etidio. ADN genómico de los juveniles transducidos con viriones pLNHX-SmActin-GFP fue empleado como molde de PCR utilizando cebadores específicos para GFP (carril 1), Neo (carril 2). Medio de cultivo del último lavado de los parásitos antes de procesarlos fue empleado como control utilizando cebadores para GFP (carril 3) y Neo (carril 4). Plásmido pLNHX-SmActin-GFP fue utilizado como molde (control positivo) y cebadores específicos para GFP (carril 5) y Neo (carril 6). Las flechas indican las bandas del tamaño esperado para ambos transgenes. <u>Panel inferior:</u> Autoradiografía de las señales de hibridación Southern de los productos de PCR, empleando una sonda marcada generada a partir de un fragmento de 4.5kb digerida con KpnI del plásmido pLNHX-SmActin-GFP que abarca los genes codificantes de resistencia a la neomicina (Neo) y GFP.
Panel C: Cuantificación por PCR en tiempo real del número de copias del transgen (luciferasa) por nanogramo de ADN extraído de juveniles de F. hepatica transducidos con MLV mediante electroporación (electroporated) o mediante la simple incubación de los parásitos en presencia de MLV ("soaked")

DISCUSION GENERAL

En la era post-genómica de la Parasitología Molecular, la información obtenida a partir de los proyectos Genomas comienza a ser analizada desde el punto de vista funcional. El desarrollo de aproximaciones experimentales que permitan avanzar en esta dirección aportará no solo a una comprensión más acabada de los procesos biológicos parasitarios, sino también en su aplicación biomédica para el desarrollo de estrategias diagnósticas y terapéuticas más efectivas (Janitz 2007). Los datos de los genomas y transcriptomas que se generan a gran escala deben analizarse empleando diversas aproximaciones. Desde el punto de vista bioinformático hay herramientas que predicen secuencias génicas, reguladoras o codificantes, realizar análisis filogenético y presumir funciones por analogía de secuencias con otros genes de función conocida de la misma u otra especie de organismo. Herramientas de genómica funcional que permitan realizar genética directa como reversa se están desarrollando. La transgénesis y la interferencia de ARN permitirán a partir de los datos de secuencia, analizar, reasignar y confirmar funciones de productos génicos. Las proteínas se pueden analizar a gran escala mediante aproximaciones proteómicas (mediante electroforesis de geles en dos dimensiones y espectrometría de masa), en particular el estudio del interactoma descubriendo proteínas que interactúan entre sí (mediante técnicas de doble hibrido de levadura), específicamente localizándolas en alguna subregión celular o analizando el conjunto de proteínas que se secretan (secretoma).

Todas estas aproximaciones orquestadas entre sí, abren el camino para detectar mediadores moleculares relevantes en la interfase huésped –parásito, que resulten útiles para generar nuevas herramientas diagnósticas (ej. como marcadores de infección), preventivas (ej. diseñando ensayos de vacunación) y/o terapéuticas (ej. diseñando nuevas drogas antiparasitarias). Una vez que se diseñe una nueva droga y/o vacuna, comienza la etapa de análisis en modelos animales, para luego transferirlas a ensayos clínicos controlados en humanos y animales de producción. La **figura 14** resume estas estrategias mencionadas aplicadas a la Parasitología Molecular.

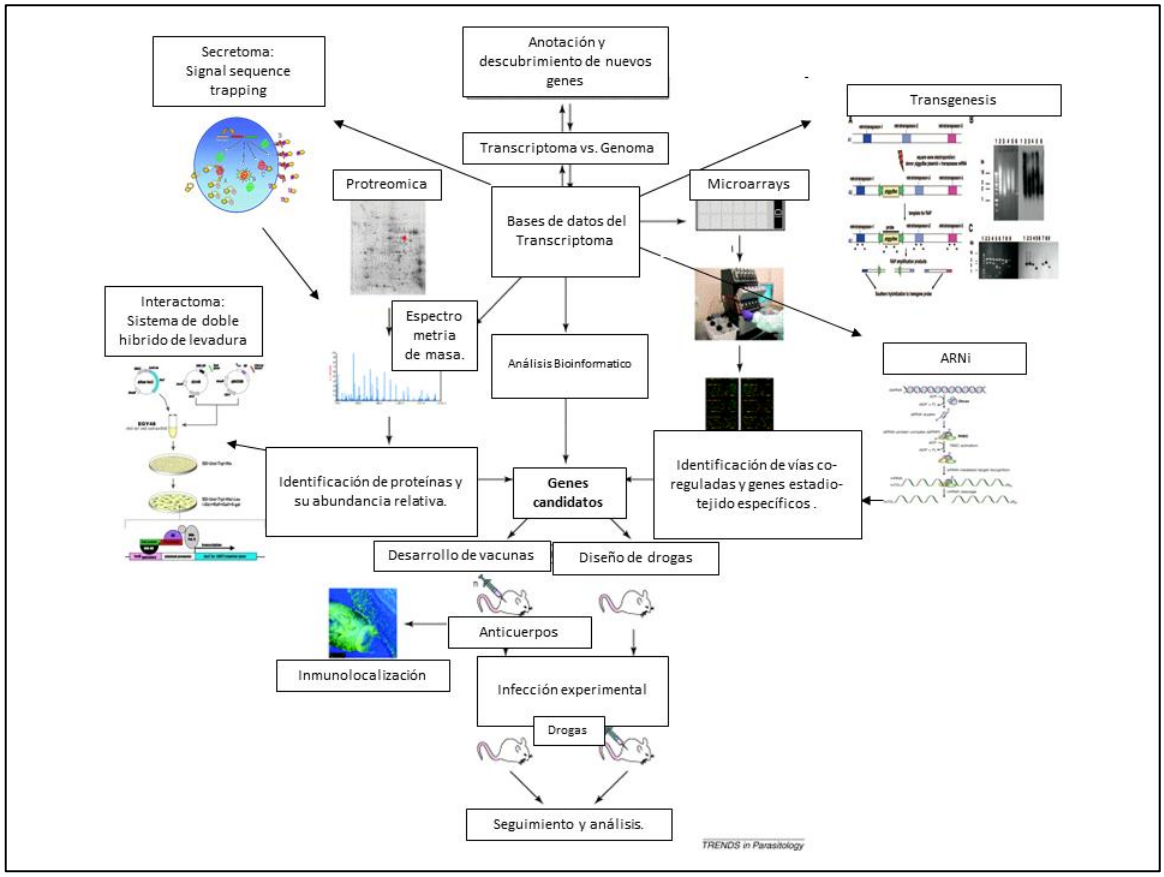

Figura 14: Herramientas de Genómica Funcional. *Aplicación de las herramientas al diseño de drogas y desarrollo de vacunas en Parasitología Molecular. Tomado y traducido de TRENDS in Parasitology.*

Nuestro interés se ha centrado principalmente en el desarrollo y optimización de herramientas de estudio de función génica, con aproximaciones de pérdida y ganancia de función en parásitos trematodos de importancia médica y veterinaria (Rinaldi 2008).

Teniendo en cuenta las particularidades del ciclo de vida de los trematodos, nos hemos planteado como hipótesis que es posible generar modificaciones genéticas estables y transgeneracionales en dichos parásitos. Para ello, focalizamos el trabajo en tres aspectos vinculados. Por un lado optimizar la transgénesis en la especie modelo *Schistosoma mansoni* desarrollando herramientas específicas para cuantificar y evaluar la

transgénesis. En segundo lugar nos interesamos en estudiar la viabilidad de transfectar las células germinales y para ello realizamos estudios de pérdida y ganancia de función en huevos, estadio donde parece más factible cumplir con dicho objetivo. Finalmente nos planteamos testar las herramientas desarrolladas y optimizadas en otras especies buscando ampliar el alcance de la genómica funcional más allá de la especie modelo.

A. Optimización de la transgénesis en *Schistosoma mansoni*

Más de 10 años han transcurrido desde los primeros trabajos de manipulación génica en parásitos del genero *Schistosoma*, y desde entonces grandes progresos se han generado. Se han transfectado moléculas reporteras mediante bombardeo de partículas y electroporación, y se ha establecido y optimizado la interferencia de ARN (ARNi) como una herramienta de rutina para asignar roles funcionales a secuencias génicas de *Schistosoma*. La transgénesis mediada por vectores que se integran al genoma ha sido probada exitosamente en *Schistosomas* (Morales et al. 2007; Kines et al. 2008). En particular, el virus de la leucemia murina modificado (MLV) ha sido empleado para transducir schistosomas quedando demostrado por análisis de secuencia que el provirus se integró al genoma de los parásitos tratados y es capaz de expresar genes reporteros (Kines et al. 2006; Kines et al. 2008). A pesar de estos reportes promisorios, la optimización de la técnica es prioritaria, quedando pendientes temas como la selección, cuantificación, localización de los eventos de integración y análisis de la expresión de los trangenes.

Con el objetivo de poder cuantificar la presencia de los transgenes integrados desarrollamos un protocolo de PCR cuantitativo anclado a retroposones o qRAP (del inglés "quantitative Retrotransposon Anchored PCR"). El mismo está basado en el Alu-PCR, una técnica desarrollada para estimar el número de copias del provirus HIV integrado en células humanas (O'Doherty et al. 2002), y en el RAP (del inglés: "Retrotransposon Anchored PCR"), una aproximación desarrollada para clonar secuencias integradas del provirus que utiliza cebadores de elementos repetidos endógenos y cebadores que hibridan con el transgen (Morales et al. 2007; Kines et al.

2008). El qRAP consiste en dos PCR consecutivos, un primer PCR convencional similar al RAP y un segundo PCR cuantitativo que utiliza como molde el producto del primer PCR y cebadores (y sonda Taqman) dirigidos contra el transgen. Con los controles y las curvas de referencias (curvas estándar) adecuadas fue posible cuantificar el número de copias del transgen integrado en el ADN genómico de parásitos transducidos con MLV.

Para validar esta técnica cuantitativa empleamos gusanos adultos de *S. mansoni* intactos y fragmentados, pero viables (donde la superficie de contacto es mayor), que fueron transducidos con la misma cantidad de viriones MLV, y detectamos mediante el qRAP un numero significativamente mayor de copias del transgen integrado en los parásitos fragmentados en comparación con parásitos intactos. La fragmentación de los gusanos permite aumentar la superficie de contacto, exponiendo tejidos más profundos a la acción del vector, resultando en un mayor número de células transducidas. Esta estrategia no es viable para la transgénesis estable, pero demuestra las dificultades de acceso a tejidos internos en organismos multicelulares. En conclusión el qRAP, permite estimar la eficiencia de transducción e integración genómica de retrovirus y otros vectores integrables.

Es interesante conocer si existen sitios preferenciales de inserción de los retrovirus en el genoma o si, por el contrario, ésta es aleatoria. De existir ciertas regiones preferidas para la inserción, ¿cuáles serían y como afectarían la expresión de los genes del provirus? Estos datos son relevantes a la hora de diseñar estrategias de terapia génica dirigida a blancos específicos mediada por vectores. Numerosos son los estudios que intentan comprender la biología básica de retrovirus y lentivirus no solo para encontrar nuevos blancos terapéuticos, sino también para optimizar los sistemas de terapia génica (Bushman *et al.* 2005).

Tres retrovirus (retrovirus de la leucemia mieloide o MLV, el virus de la sarcomatosis-leucosis aviar o ASLV, y el virus de la inmunodeficiencia humana o HIV) se han estudiado en detalle y muestran patrones diferenciales en lo que respecta a la preferencia de los sitios de integración genómica. En células humanas MLV se distribuye

a lo largo de todo el genoma pero tiende a integrarse en las regiones 5' de los genes, cercanas al sitio de inicio de la transcripción. Asimismo se ha demostrado que MLV se inserta preferencialmente en islas CpG, sitios hipersensibles a la ADNsa I y sitios de unión a factores de transcripción, por lo que regiones transcripcionalmente activas serían blanco de integración de MLV (Lewinski et al. 2006). Aunque HIV se distribuye también globalmente en todo el genoma de las células humanas, se ha observado un sesgo hacia las unidades transcripcionales, pero a diferencia de MLV sin ninguna región preferencial dentro de la unidad transcripcional. Finalmente, el virus ASLV ha mostrado un patrón de integración genómica más aleatorio. Aunque algunos reportes señalan que las regiones transcripcionalmente activas serían sitios desfavorecidos para la integración de ASLV, más estudios hacen falta para aclarar este punto (Bushman et al. 2005).

El estudio a gran escala de los sitios de integración de MLV en el genoma de *Schistosoma mansoni* no solo permitirá demostrar la utilidad de estos vectores en la generación de líneas de parásitos transgénicos, sino también comprender mejor los mecanismos básicos que subyacen el ciclo de vida de los retrovirus en general. Con la intensión de generar datos que permitan conocer los sitios preferenciales de integración de MLV en schistosomas, transducimos diversos estadios de desarrollo de *S. mansoni* (adultos, schistosomulas y huevos) con MLV y analizamos los sitios de integración del retrovirus en el ADN genómico mediante aproximaciones a gran escala utilizando secuenciación de nueva generación Illumina. Los resultados preliminares, muestran que en 1248 eventos de integración estudiados no se observa ningún sesgo de integración en el genoma de los parásitos transducidos. Los porcentajes de los eventos de integración detectados en regiones no codificantes, exones, intrones y UTRs no se diferencian significativamente con los porcentajes representados por estas regiones en el genoma de *S. mansoni* (Berriman et al. 2009). A pesar de que hemos utilizado una técnica de secuenciado masivo que es capaz de generar millones de lecturas de secuencia, fueron relativamente pocos los eventos de integración detectados. Esto estaría denotando una baja eficiencia de la transducción viral de parásitos, una reducida integración del provirus en el genoma, o ambas. Es probable que en las células de schistosomas, a diferencia de las células de mamífero, estén ausentes factores proteicos relevantes para completar el

ciclo de vida del retrovirus lo que determina una disminución de la cantidad efectiva de provirus integrado al genoma del parásito. Más estudios en esta dirección son necesarios.

Una aproximación factible para incrementar el número de copias del transgen en schistosomas transducidos con MLV, y así poder recuperar una mayor cantidad de eventos de integración del provirus, es la selección de organismos transgénicos mediante un marcador de resistencia a una droga (por ej. antibiótico). Los sistemas de selección genética que utilizan antibióticos en combinación con genes de resistencia a esos antibióticos expresados en los organismos que se quieran rescatar/seleccionar son técnicas imprescindibles en los laboratorios actuales de biología molecular y de rutina cuando se utilizan bacterias y células eucariotas genéticamente modificadas. De esta manera la población de organismos transgénicos que expresa un gen de resistencia a antibiótico es enriquecida. Hasta el año pasado no existían reportes que demostraran la factibilidad de estas estrategias de selección en helmintos. En el 2010 se reportó el empleo de antibióticos (geneticina y puromicina) para rescatar *Caenorhabditis elegans* transgénicos que expresan genes de resistencia a dichos antibióticos (Giordano-Santini *et al.* 2010; Semple *et al.* 2010).

Puesto que se ha visto que los transgenes codificados en el genoma del virus de *la* leucemia murina modificado (MLV) se expresan activamente en *Schistosoma* (Kines *et al.* 2008), nos preguntamos si era posible enriquecer la población de schistosomas trangenicos basándose en la selección con geneticina de parásitos transducidos con MLV codificantes del gen *neoR*, de resistencia al antibiótico Geneticina (G418).

En primer lugar definimos las condiciones de selección, estableciendo la sensibilidad del parásito al antibiótico mediante curvas de sobrevida. Se testaron diferentes concentraciones de antibiótico y tiempos de cultivo y se determinó la vitalidad por diversos métodos. Se comenzaron a observar efectos tóxicos evidentes a partir del sexto día de cultivo, con diferencias significativas en la vitalidad de los parásitos en comparación con controles cultivados en ausencia de la droga. Posteriormente evaluamos la capacidad de MLV portador en su genoma del gen *neoR* para rescatar schistosomulas

transducidas y cultivadas en presencia de G418. En todas las concentraciones testadas se observó que los parásitos transducidos con MLV sobrevivieron más que los controles no tratados en presencia del antibiótico. Estas observaciones fenotípicas fueron respaldadas por análisis moleculares que demostraron la presencia y la expresión del transgen en la población de schistosomulas transducidas con el virus. La correlación entre el número de copias detectado en el ADN de parásitos tratados y la concentración de G418 a la que fueron expuestos, estaría reflejando un enriquecimiento de la población de parásitos transgénicos.

Si bien nuestra aproximación es funcional en schistosomas y permite enriquecer la población de parásitos transgénicos, el efecto reportado para *C. elegans* fue mucho más acentuado. El porcentaje de gusanos transgénicos obtenidos cuando se cultivaron en presencia de la droga puromicina fue al menos 50 veces mayor al porcentaje de gusanos transgénicos recuperados al cultivarlos en ausencia del antibiótico (Semple *et al.* 2010). En esta estrategia los autores trabajaron con gusanos en los que se espera que la mayoría de las células sino todas sean portadoras del transgen, ya que derivan de células de la línea germinal transfectadas con el vector plasmídico codificante del gen de resistencia. A diferencia de la selección de *C. elegans*, donde la transgénesis es de la línea germinal, en nuestro sistema es esperable que solo algunas células sean transducidas por MLV, probablemente células del tegumento o del tubo digestivo (transgénsis somática), y por lo tanto la selección de los parásitos es mucho menor. Por otro lado, el nivel de expresión del gen *neoR* debe ser lo suficientemente elevado y mantenido en el tiempo como para lograr un efecto de rescate de las células transducidas y consecuentemente del organismo en su conjunto. Un nivel bajo de expresión del gen *neoR* puede también explicar la diferencia entre el efecto de enriquecimiento detectado en nuestro sistema comparado con el reportado para *C. elegans* (Semple *et al.* 2010).

Otra estrategia de selección de helmintos modificados genéticamente y testada en *Caenorhabditis elegans*, es el clasificador de gusanos (del inglés “worm sorter”), “citómetro” para organismos multicelulares que permite seleccionar gusanos que expresen un gen reportero determinado, y de esta forma enriquecer una población de

gusanos transgénicos (Rohde *et al.* 2007). La ventaja de esta técnica de clasificación de gusanos es que no depende de un gen de resistencia a antibiótico que debe mantener un nivel de expresión adecuado que permita rescatar los organismos transducidos en presencia del antibiótico en cuestión.

En sistemas retrovirales empleados en terapia génica se ha reportado una expresión variable e incluso silenciamiento de los transgenes integrados en el genoma (Malik and Arumugam 2005). Por este motivo nos interesamos en estudiar si en nuestro sistema se observaba un fenómeno similar de silenciamiento de *neoR*, y de ser así, si era factible modificar nuestros vectores retrovirales para asegurar una expresión más elevada y estable en el tiempo.

Para ello modificamos el vector plasmídico utilizado para generar el virus de la leucemia murina modificado (MLV) insertando en la secuencia del elemento aislante de la cromatina cHS4 (del inglés "chicken hypersensitive site 4"). cHS4 es un elemento aislante de la cromatina presente en el locus de la β-globina de pollo que participa en la regulación de la expresión génica de las globinas bloqueando las interacciones entre promotores y potenciadores. Ha sido utilizado para proteger la expresión de transgenes en *Drosophila*, líneas celulares y en mamíferos transgénicos de los efectos de silenciamiento posicional de la cromatina (Chung *et al.* 1993; Emery *et al.* 2000).

El perfil de expresión temporal del *neoR* fue analizado en schistosomulas transducidas con virus MLV con y sin el aislante cHS4. La expresión global del gen *neoR* disminuyó en el tiempo en ambos grupos, sin embargo la reducción observada en el grupo de schistosomulas tratadas con el virus con cHS4 fue mucho menor, lo que sugiere que el elemento aislante de la cromatina presente en el vector retroviral logró mantener una mayor expresión del gen *neoR*. A pesar de esto, se detectó un silenciamiento progresivo del transgen en ambos grupos, fenómeno que deberá ser estudiado en profundidad en el futuro. Obtener la máxima expresión de los transgenes insertos en los vectores retrovirales minimizando los fenómenos de silenciamiento progresivo, es un

objetivo fundamental en el desarrollo de la transgénsis estable y transgeneracional que redundará en el establecimiento de líneas de parásitos genéticamente modificados.

Recientemente se ha reportado que el genoma de *Schistosoma mansoni* presenta metilación (Geyer *et al.* 2011), lo que sugiere la posibilidad de eventos epigenéticos de regulación de la expresión génica en estos parásitos. Nos preguntamos si estos fenómenos de regulación génica a nivel de la cromatina son los que están afectando la expresión de los transgenes retrovirales, y de ser así cuales serían los mecanismos involucrados. El estudio del estado de metilación específicamente de los transgenes retrovirales (con o sin elemento aislante de cromatina) permitirá responder alguna de estas preguntas, y permitir en un futuro diseñar vectores retrovirales más eficientes en cuanto a la expresión de los transgenes.

B. Evaluación de la transfección estable de células germinales

La generación de líneas de *Schistosomas* genéticamente modificados mediante transgénesis estable y transgeneracional será posible si se transducen las células germinales responsables de la amplificación clonal en el huésped intermediario. Para ello es necesario transfectar huevos o miracidios, pues presentan una alta relación células germinales/células somáticas con lo que son más altas las probabilidades de transducir células de la línea germinal (Jurberg *et al.* 2009).

Al definir a los huevos como un estadio de desarrollo potencial para establecer la transgénesis estable y transgeneracional la primera pregunta planteada fue si era factible manipular genéticamente este estadio. Para ello estudiamos la factibilidad del silenciamiento génico mediante interferencia de ARN (ARNi), y el uso de moléculas reporteras en este estadio.

Una primera aproximación fue el silenciamiento temporal mediante la inoculación de ARN de doble cadena dirigidos contra dos proteasas que aparecían como relevantes en huevos. La actividad de enzimas leucinaminopeptidasas (LAPs) fue detectada en huevos

de *Schistosoma manosni* hace más de 30 años (Bogitsh and Carter 1975; Bogitsh 1983) y estudios histoquímicos más recientes localizaron LAPs en huevos y en el infiltrado inflamatorio producido por los huevos en el tejido hepático de ratones infectados experimentalmente (Abouel-Nour *et al.* 2005). Por otro lado existía evidencia experimental de que la actividad LAP en huevos podría estar vinculada al proceso de eclosión de los mismos, ya que el tratamiento de huevos con bestatina (inhibidor de las LAPs) redujo significativamente la emergencia de los miracidios (Xu and Dresden 1986).

La disponibilidad del genoma de *S.mansoni* nos permitió verificar la existencia de dos genes de LAP, que se expresan en varios estadios del parásito, aunque la LAP 2 se expresa con mayor intensidad que la LAP 1 en miracidio y estadios intra-caracol, lo que podrá estar sugiriendo una especialización funcional de esta enzima en estos estadios. El tratamiento con ARNdc contra LAPs por separado o en conjunto resultó en una notoria disminución de la eclosión de miracidios, similar a la observada con inhibidores. Este efecto fue acompañado por una disminución específica del ARNm de la enzima silenciada, así como una reducción en la actividad enzimática de la LAP medida en extractos de los huevos tratados. De esta manera utilizando interferencia de ARN aportamos evidencias que sugieren una vinculación funcional entre las LAPs de *Schistosoma mansoni* y el proceso de eclosión, lo que posiciona a estas enzimas como blancos potenciales para el desarrollo de nuevas drogas o vacunas. Por otra parte, y quizás más importante en relación a nuestro actual proyecto, es que fuimos capaces de manipular exitosamente la expresión génica en huevos de *Schistosoma mansoni*.

Una vez que demostramos que fue posible alterar la expresión génica en huevos mediante ARNi, nos preguntamos si eran factibles otras estrategias de manipulación genética en este estadio de desarrollo. En primer lugar transfectamos huevos mediante electroporación o la simple incubación de los huevos en presencia de dos tipos de moléculas reporteras. Utilizamos pequeños ARNs doble cadena marcados con el fluorocromo Cy3 (del inglés “short interfering RNAs” o siRNAs) y ARNm de la luciferasa de luciérnaga, y en ambos casos evidenciamos la incorporación de las moléculas reporteras en los huevos. Posteriormente empleamos partículas virales MLV

para introducir transgenes en el interior de los huevos, los cuales al cabo de pocos días se eclosionaron y los miracidios fueron colectados y procesados para evaluar la presencia del provirus mediante PCR convencional. El provirus fue detectado en el ADN de miracidios provenientes ya sea de huevos electroporados o simplemente incubados en presencia de MLV. Al menos dos veces más copias del transgen (gen de la luciferasa) fueron detectadas en el ADN de los miracidios provenientes de los huevos electroporados en presencia del virus. Concluimos que es factible transfectar diferentes moléculas reporteras en huevos de *Schistosoma mansoni*, y además detectamos que la electroporación es más eficiente para la incorporación de transgenes en el ADN de los parásitos transfectados en este estadio de desarrollo.

Estos resultados promisorios indicaron que es posible manipular huevos, incrementando las probabilidades de transfectar células de la línea germinal con vectores integrables en el genoma. Una vez transfectados los huevos, se pueden eclosionar, utilizar los miracidios resultantes para infectar caracoles y las cercarias emitidas por dichos caracoles pueden ser analizadas buscando la presencia del transgen en su genoma. En este sentido, Grevelding y colaboradores pudieron transfectar células de la línea germinal en miracidios bombardeados con plásmidos reporteros. La presencia y expresión del gen reportero fue confirmada en cercarias y adultos de las generaciones F0 y F1, perdiéndose a partir de la F2 (Beckmann *et al.* 2007). Al no ser un vector integrable, probablemente el plásmido se perdió y/o diluyó a lo largo de las generaciones.

Una vez que verificamos que es factible introducir transgenes mediante la transducción de huevos con MLV procedimos a infectar caracoles con los miracidios obtenidos a partir de huevos transducidos, y mediante una técnica de secuenciado a gran esacala (Illumina) detectamos la presencia del provirus en el ADN de cercarias emitidas por dichos caracoles. Estos resultados preliminares, mostrados en la **figura 12** (*sección resultados*), son promisorios y estarían indicando que es factible la transmisión de transgenes de un estadio a otro del desarrollo del parásito (en este caso de huevo/miracidio a cercaria). Con esas cercarias es posible infectar experimentalmente

ratones para propagar el ciclo del parásito y generar una línea transgénica de *Schistosomas* que se puede mantener en el laboratorio.

La generación de líneas de schistosomas modificados genéticamente en forma estable permitirá contar con parásitos que expresen en forma constitutiva, tejido-específica o en forma condicional, dependiendo del promotor empleado, genes reporteros, secuencias codificantes de interés o cassettes de expresión de horquillas de ARN (del inglés "short hairpin RNAs" o shRNAs) que induzcan silenciamiento génico mediado por interferencia de ARN. La interferencia de ARN convencional mediada por ARN doble cadena determina un efecto de silenciamiento génico post-transcripcional que por lo general es transitorio, de intensidad variable dependiendo de la secuencia elegida para generar el ARNdc o del gen que se quiere silenciar. Como hemos visto, la accesibilidad a algunos tejidos internos puede ser un problema en estos organismos, por lo que algunos genes pueden ser refractarios al silenciamiento por ARNi, o pueden inducir silenciamiento inespecífico por inducción de la respuesta del interferón y apoptosis (Haasnoot *et al.* 2007; Sliva and Schnierle 2010; Handa *et al.* 2011). El empleo de vectores que incluyan cassettes codificantes de shRNAs puede llegar a sortear estos inconvenientes inherentes al ARNi convencional.

Recientes reportes demuestran que es posible inducir silenciamiento génico en *Schistosomas* mediante el empleo de horquillas de ARN (del inglés "short hairpin RNAs" o shRNAs) codificados en vectores plasmídicos o retrovirales (Zhao *et al.* 2008; Tchoubrieva *et al.* 2010). Zhao y colaboradores empleando shRNAs codificados en plásmidos bajo el control de un promotor de la ARN pol III, logran un silenciamiento significativo con un evidente efecto fenotípico, pero con un efecto eventualmente transitorio (Zhao *et al.* 2008). Tchoubrieva por el contrario emplea virus de la leucemia murina modificado (MLV) codificante de una horquilla dirigida contra la enzima catepsina B1 de *S. mansoni* bajo el promotor de la actina (un promotor de ARN pol II), observando también silenciamiento (Tchoubrieva *et al.* 2010).

En nuestro caso colocamos un shRNA dirigido contra el gen reportero luciferasa bajo el control del promotor del gen U6 de *S.mansoni* y una construcción similar con el mismo promotor de ARN Pol III de origen humano. La transfección de schistosomulas con los vectores integrables *piggyBac* y MLV conteniendo el cassette silenciador seguida de la incorporación del ARNm codificante para la luciferasa resultó en una reducción de la actividad enzimática de la luciferasa. El efecto fue repetido en líneas celulares humanas con resultados similares a los hallados en *S. mansoni*. Asimismo, comparando la eficiencia de los promotores del gen U6 humano y el de *Schistosoma* para inducir silenciamiento de la luciferasa, observamos que el promotor U6 humano indujo una mayor reducción de la actividad luciferasa que su ortólogo de *Schistosoma*. La existencia de múltiples copias y variantes del gen U6, tanto en humanos como en nuestro modelo parásito puede aportar una explicación a estas diferencias. El promotor humano fue obtenido de un kit comercial (Clontech) ya optimizado para inducir ARNi mediado por shRNAs, mientras que desconocemos si la variante del promotor U6 de *Schistosoma* utilizada es funcional o la más activa o funcional.

C. Transferencia de tecnología a otros parásitos trematodos

La genómica funcional en otros trematodos diferentes a los modelos *S.mansoni* y *S.japonicum* es apenas incipiente. En el año 2008 probamos que es posible transferir la tecnología previamente desarrollada en *S. mansoni* a *Fasciola hepatica,* generando un protocolo sencillo para evaluar la presencia de una vía activa de ARNi mediante el silenciamiento con ARN doble cadena de un gen reportero (luciferasa de luciérnaga) (Rinaldi *et al.* 2008). Esta herramienta ha sido extendida a otros trematodos como *Opistorchis viverrini*, y cestodos como *Moniezia expansa* y *Echinococcus multilocularis* (Pierson et al. 2009; Mizukami et al. 2010; Sripa et al. 2011).

El impacto en salud pública producido por *Schistosoma haematobium*, agente responsable de la schistosomiasis urinaria, supera al producido por *S. mansoni* y *S. japonicum* combinados. Además la infección por *S. haematobium* se asocia con (1) el carcinoma escamoso de vejiga y (2) el aumento en la susceptibilidad de la infección por

HIV. A pesar de esto, es la especie menos estudiada desde el punto de vista molecular, no se cuenta aún con el genoma completo y no se han desarrollado aún las herramientas de estudio de función génica (Rollinson 2009). Consecuentemente intentamos transferir algunas de las herramientas de genómica funcional bien establecidas en *S. mansoni* a este modelo.

En primer lugar obtuvimos diferentes estadios de desarrollo del parásito y logramos cultivarlos siguiendo condiciones previamente establecidas para *S. mansoni* (Mann et al. 2010). Utilizando pequeños ARNs doble cadena (siRNA) marcados con el fluorocromo Cy3 y ARNm de luciferasa, logramos transfectar distintos estadios de desarrollo de *S. haematobium* mediante electroporación o simplemente incubando los parásitos en presencia de la molécula reportera. Siguiendo el protocolo desarrollado en *S.mansoni* y testado en *F.hepatica,* logramos evidenciar la presencia de una vía activa de ARNi en *S.haematobium*. Esto fue detectado usando un reportero exógeno y un gen endógeno, la tetraspanina 2, antígeno candidato al desarrollo de vacunas.

Finalmente buscamos extender el uso de vectores virales modificados como agentes de transducción en otras especies, utilizando para ello juveniles de *Fasciola hepatica*. Luego de la transfección con MLV con distintos reporteros de juveniles en cultivo fue posible detectar la presencia del transgen en el ADN genómico extraído de los parásitos tratados. Estos resultados preliminares abren promisorias perspectivas al desarrollo de herramientas de transgénesis estable en este trematodo de importancia veterinaria y de gran impacto en nuestro país.

CONCLUSIONES Y PERSPECTIVAS

Los objetivos planteados originalmente han sido satisfactoriamente cumplidos. En primer lugar comenzamos a optimizar la técnica de transgénesis ya desarrollada en el parásito modelo *Schistosoma mansoni*. En este sentido establecimos una técnica cuantitativa de estimación del número de transgenes integrados en el genoma de los parásitos transfectados y comenzamos a analizar a escala genómica los sitios de integración del virus de la leucemia murina modificado (MLV) en el genoma de *S. mansoni*. Asimismo establecimos las condiciones de selección mediante antibiótico de parásitos transgénicos y analizamos el perfil temporal de expresión de transgenes en vectores virales que contienen secuencias aislantes de la cromatina con el objetivo de contrarrestar potenciales efectos de silenciamiento posicional.

En segundo lugar, con el objetivo de evaluar la posibilidad de generar un sistema de transgénesis estable y transgeneracional, analizamos la capacidad de transducir células de la línea germinal presentes en los huevos del parásito. Logramos manipular la expresión génica de huevos de *S. mansoni* mediante la interferencia de ARN doble cadena, y transfectamos en este estadio de desarrollo, moléculas reporteras, incluyendo MLV. Resultados primarios mostraron que los transgenes incorporados en los huevos del parásito mediante la transfección con MLV, fueron exitosamente transmitidos a través de los estadios intracaracol, estableciendo la posibilidad de generar en un futuro líneas estables de *Schistosomas* transgénicos.

Colaboramos en el diseño y desarrollo experimental de aproximaciones de interferencia de ARN no convencional, mediada por vectores codificantes de horquillas de ARN, en *S. mansoni*.

Finalmente logramos transferir exitosamente algunas herramientas de manipulación génica a *S. haematobium*, la especie del género *Schistosoma* más desatendida, y comenzamos a desarrollar la transgénesis en *Fasciola hepatica* otro trematodo con un impacto particular en Uruguay.

Como resultado de este trabajo se generaron más preguntas que respuestas, que serán indagadas en el futuro. Para seguir optimizando la transgénesis en el modelo *S. mansoni* debemos analizar en profundidad los sitios de integración del provirus MLV en el genoma, y como podría afectar la región de la cromatina y los eventos epigenéticos, recientemente descubiertos en *Schistosoma*, a la integración y a la expresión de los transgenes. Debemos seguir optimizando la selección de parásitos transgénicos mediante antibióticos u otras aproximaciones, actualmente disponibles para otros modelos.

El silenciamiento génico estable mediante la incorporación de cassettes productores de horquillas de ARN interferente bajo el control de distintos promotores, incluyendo promotores condicionales, deberá seguir siendo desarrollado y optimizado.

Finalmente, pero no menos importante, la transferencia de tecnología de genómica funcional a otros parásitos helmintos, como vimos con *S. haematobium* o *F. hepatica* es una prioridad. Platelmintos parásitos donde la manipulación génica se encuentra en etapas iniciales, como *Echinococcus granulosus* o *Mesocestoides corti*, podrían ser testados. Estos parásitos son importantes ya sea por la aún alta morbimortalidad de su infección, caso de *E. granulosus*, o como en el caso de *M. corti*, por ser modelos de estudio de referencia en Parasitología Molecular.

Estas herramientas contribuirán no solo a la comprensión de la biología básica de las parasitosis, sino que definirán implicancias funcionales de mediadores de la interfase huésped-parasito, blancos potenciales de nuevas estrategias de control parasitario.

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ANEXO I

Se incluyen dos artículos en los que he participado como colaborador durante el transcurso de este trabajo.

- shRNAs expresados bajo el control del promotor del gen U6 de *Schistosoma mansoni* induce ARN de interferencia en células de fibrosarcoma humanas y schistosomulas

  Ayuk MA*, Suttiprapa S*, **Rinaldi G**, Mann VH, Lee CM, Brindley PJ
  (* Igual contribución al trabajo)

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- El promotor humano U6 promueve una mayor actividad de shRNA que su ortólogo de schistosoma en *Schistosoma mansoni* y células de fibrosarcoma humanas

  Raphaël Duvoisin *, Mary A. Ayuk *, **Gabriel Rinaldi**, Sutas Suttiprapa, Victoria H. Mann, Clarence M. Lee, Nicola Harris, Paul J. Brindley
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Schistosoma mansoni U6 gene promoter-driven short hairpin RNA induces RNA interference in human fibrosarcoma cells and schistosomules ☆

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Promoter

ABSTRACT

RNA interference (RNAi) mediated by short hairpin-RNA (shRNA) expressing plasmids can induce specific and long-term knockdown of specific mRNAs in eukaryotic cells. To develop a vector-based RNAi model for *Schistosoma mansoni*, the schistosome U6 gene promoter was employed to drive expression of shRNA targeting reporter firefly luciferase. An upstream region of a U6 gene predicted to contain the promoter was amplified from genomic DNA of *S. mansoni*. A shRNA construct driven by the predicted U6 promoter targeting luciferase was assembled and cloned into plasmid pXL-Bac II, the construct termed pXL-Bac-II_SmU6-shLuc. Luciferase expression in transgenic fibrosarcoma HT-1080 cells was significantly reduced 96 h following transduction with plasmid pXL-BacII_SmU6-shLuc, which encodes luciferase mRNA-specific shRNA. In a similar fashion, schistosomules of *S. mansoni* were transformed with the SmU6-shLuc or control constructs. Firefly luciferase mRNA was introduced into transformed schistosomules after which luciferase activity was analyzed. Significantly less activity was present in schistosomules transfected with pXL-BacII_SmU6-shLuc compared with controls. The findings revealed that the putative *S. mansoni* U6 gene promoter of 270 bp in length was active in human cells and schistosomes. Given that the U6 gene promoter drove expression of shRNA from an episome, the findings also indicate the potential of this putative RNA polymerase III dependent promoter as a component regulatory element in vector-based RNAi for functional genomics of schistosomes.

1. Introduction

Draft genomes of *Schistosoma japonicum* and *Schistosoma mansoni* were reported recently (Berriman et al., 2009; *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium, 2009). New information in genome sequences will provide leads for development of new interventions for control and treatment of schistosomiasis (Brindley et al., 2009), which relies solely of the anthelmintic drug praziquantel at this time. Tools are in development to determine the importance of the new genomic sequences of schistosomes (Beckmann et al., 2007; Brindley and Pearce 2007; Kines et al., 2008; Mann et al., 2008), although there are difficulties with investigating the complex genomes of helminth parasites such as the schistosomes in contrast to model, free-living species. RNA interference (RNAi) is active in schistosomes, has been used to investigate increasing numbers of gene targets in most developmental stages, and is being optimized for high-throughput screens (Stefanic et al., 2010 and references therein).

Despite its utility, RNAi frequently leads to only 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 small interfering RNAs (siRNAs) can circumvent deficiencies with exogenous RNAi approaches by providing continuous and/or conditional gene silencing (ter Brake et al., 2006; Sliva and Schnierle, 2010). Recently, it has been demonstrated that pseudotyped murine leukemia virus (MLV) can be employed to transduce developmental stages of schistosomes, leading to chromosomal integration of retroviral transgenes and transgene reporter activity (Kines et al., 2008, 2010; Yang et al., 2010). Moreover, a vector-based RNAi approach has been reported in which the MLV transgene encoded a long hairpin RNA specific for a schistosome protease involved in hemoglobinolysis (Tchoubrieva et al., 2010).

In insect, mammalian, avian and some pathogenic protozoa, RNA polymerase III (Pol III) promoter-based DNA vectors can express

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siRNA or short hairpin RNA (shRNA) (Brummelkamp et al., 2002; Gou et al., 2003; Wakiyama et al., 2005; Linford et al., 2009). However, this has not been demonstrated in schistosomes. Here we describe a promoter-like element from the U6 gene of *S. mansoni*, a Pol III, non-coding RNA gene that is a component of eukaryotic spliceosome machinery (see Copeland et al., 2009). An episomal plasmid that included a U6 gene promoter driving a short hairpin transcript designed to target mRNA encoding firefly luciferase was constructed. After transfection of human fibrosarcoma cells and schistosomules with the construct, significantly less reporter luciferase activity was seen in the transformed human cells and schistosomes. These findings demonstrated the activity of a 270 bp promoter-like sequence of the schistosome U6 non-coding RNA gene to drive transcription of short transcripts from episomes delivered to both human cells and schistosomules of *S. mansoni*. The findings indicate the likely utility of this U6 gene promoter containing construct for vector-based RNAi approaches in schistosomes.

2. Materials and methods

2.1. Schistosomules

Biomphalaria glabrata snails infected with the NMRI (Puerto Rican) strain of *S. mansoni* were supplied by Dr. Fred Lewis, Biomedical Research Institute, Rockville, MD, USA. Cercariae released from infected *B. glabrata* snails were mechanically transformed into schistosomules. Briefly, cercariae were concentrated by centrifugation (425*g*/10 min) and washed once with schistosomule wash medium, RPMI 1640 supplemented with 1x penicillin, streptomycin, fungizone (1 × PSF) and 10 mM HEPES (Mann et al., 2010). Cercarial tails were sheared off by 20 passes through 22 gauge emulsifying needles after which schistosomule bodies were isolated from tails by Percoll gradient centrifugation (Lazdins et al., 1982). Schistosomula were washed three times in wash medium and cultured for up to 2 weeks at 37 °C under 5% CO_2 in air in modified Basch's medium (Basch, 1981) supplemented with washed human erythrocytes, at a density of 1 μl packed red cells per ml of culture medium (Mann et al., 2010).

2.2. Isolation of a U6 gene promoter-like sequence

The sequence and predicted structure of the U6 small nRNA of *S. mansoni*, GenBank accession number L25920, have been described (Gu and Reddy, 1994; Copeland et al., 2009). The 109 nucleotides (nt) of the U6 RNA were employed as the query to search the Sanger Institute's *S. mansoni* shotgun reads (Sanger) database, (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_mansoni). An identical match was located to sequences in contig shisto3397e03.p1k. Flanking sequences upstream of the copy of the U6 gene in this contig were targeted for PCR, using primers SmU6F, 5′-TATCAGTGGTCTAGATGTATGCTTG-3′, and SmU6R, 5′-GCTAATC TTCTCTGTATCGTTCCA-3′. The genome of *S. mansoni* includes between nine and 55 copies of the U6 gene (Copeland et al., 2009). Sequences (~474 bp) upstream and flanking the U6 gene as well as part of the 5′-terminus of the U6 gene were amplified from genomic DNA of *S. mansoni* (prepared as in Morales et al., 2007), cloned into the TOPO TA plasmid (Invitrogen) and the sequence identity confirmed to be identical to that in contig shisto3397e03.p1k.

2.3. Construction of shRNA expression vectors targeting firefly luciferase

The two-step PCR approach of Gou et al. (2003) and Linford et al. (2009) was utilized to construct plasmid vectors for in vivo episomal RNAi. The approach involves two rounds of PCR using one universal primer, specific for the promoter and two unique target sequences; the first oligonucleotide to add the sense strand of the shRNA, the second to add the loop region and anti-sense strand. For the hairpin sense strand sequence, we utilized 21 residues, nt 851–871, (5′-GTGCGCTGCTGGTGCCAACCC-3′-), of the firefly luciferase gene in pGL3 because this site and target sequence length have been demonstrated in earlier reports of episomal vector RNAi to lead to complete or near complete knockdown of luciferase activity in *Drosophila* Schneider 2 cells, NIH-3T3 mouse fibroblasts and L2 rat lung epithelia cells (Gou et al., 2003; Wakiyama et al., 2005). Two rounds of PCR were employed to generate the final shRNA constructs, using forward primer U_6SF_1 , 5′-GCGCGCGGATCCGAGTGTATGTGCATTTGGTTG-3′, and two reverse primers, Luc851R1, 5′-TCTCTTGAAGGGTTGGCACCAGCAGCGCAC GGATTT CGCAC ATCACTAAC-3′ and Luc851R2, 5′-GCGCGCCTCGA GAAAAAGTGCGCTGCTGG TGCCAA CCCTCTCTTGAA-3′. The final PCR product comprised the *S. mansoni* U6 promoter, the sense strand of the shRNA hairpin, a loop TTCAAGAGA (9 bp) (Brummelkamp et al., 2002) region, the antisense strand of the hairpin, and the U6 termination sequence, TTTTT (Gou et al., 2003). In addition, *Bam*HI (GGATCC) and *Xho*I (CTCGAG) sites were introduced upstream of the promoter and after the TTTTT terminator, respectively, to facilitate cloning. As a control vector, we constructed a cassette where the 21 target residues (nt 851–871) of the luciferase gene were 'scrambled' as follows, 5′-ACCTACTGGGCAGGGA GCCGC-3′, using the same forward primer (above) and the following reverse primers, ScramLuc851 R1, 5′-TCTCTTGAAA CCTAC TGGGCAGGGAGCCGCGGATTTCGCACATCACTAAC-3′; ScramLuc851 R2, 5′-GCGCGCCTCGAGAAAAAGCGGCTCCCTGCCCAGTAG GTTCTCT TGAA-3′. Thermal cycling conditions and reaction volumes of Linford et al. (2009) were employed.

The final products were sized by agarose gel electrophoresis, eluted from the gel, cleaved with *Bam*HI and *Xho*I, and ligated into linearized plasmid pXL-BacII at the multiple cloning site within the transposon's inverted terminal repeats (Li et al., 2005). Top10 *Escherichia coli* cells (Invitrogen) were transformed with the ligation products and transformed colonies cultured on Luria–Bertani broth (LB) agar-ampicillin (100 μg/ml). Maxipreps of plasmid DNA were prepared from single bacterial colonies using PerfectPrep Endofree Maxi Kit (5Prime, Gaithersburg, MD, USA), and concentration and purity determined with a spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). The nt sequences of the inserts of the vector RNAi constructs, termed pXL-BacII_SmU6-shLuc and pXL-BacII_SmU6-shScramLuc were confirmed, and assigned GenBank accession numbers HQ677838 and HQ677839, respectively.

2.4. Preparation of siRNAs, double stranded RNAs (dsRNAs) and firefly luciferase mRNA

Block-iT™ siRNA of 21 nt in length, specific for residues 851–871 of firefly luciferase (siLuc), was purchased from Invitrogen (Carlsbad, CA, USA). Long dsRNA specific for, and spanning the full-length luciferase coding sequence of 1672 bp was synthesized by in vitro transcription from a template amplified by PCR from the firefly luciferase gene in pGL3 (Promega), using primers (F, 5′-TAA TAC GAC TCA CTA TAG GG T GCG CCC GCG AAC GAC ATT TA-3′ and R, 5′-TAA TAC GAC TCA CTA TAG GGG CAA CCG CTT CCC CGA CTT CCT TA-3′) tailed with the T7 promoter sequence). Synthesis and downstream purification of the dsRNA was accomplished using the Megascript RNAi kit (Ambion, Austin, TX, USA). Integrity of dsRNAs was verified visually after non-denaturing agarose gel (1%) electrophoresis. For preparation of firefly luciferase mRNAs (mLuc), in vitro transcriptions of capped RNAs from template PCR products were accomplished using the mMessage mMachine T7 Ultra kit (Ambion) (Correnti and Pearce, 2004; Rinaldi et al., 2008). dsRNA

or mLuc was precipitated with 1 vol. of 5 M ammonium acetate and 2.5 vol. of 95% ethanol after which the precipitate was dissolved in water and concentration and purity determined, as above.

2.5. HT1080 cells expressing firefly luciferase

We employed a genetically modified HT1080 human fibrosarcoma cell line that constitutively expressed luciferase in these studies of vector-based RNAi. Primarily for other studies (Suttiprapa, Mann and Brindley, unpublished data), we constructed lentiviral vectors using the ViraPower Gateway system (Invitrogen). In brief, pLenti6/R4R2/V5-DEST, which includes a gene conferring resistance to blasticidin, was modified by insertion of the promoter of the *S. mansoni* spliced leader RNA gene, upstream of the firefly luciferase gene (see Kines et al., 2006). The 293FT producer cells (Invitrogen) were transformed with the construct together with packaging plasmids delivered in liposomes (Invitrogen). Pseudotyped lentivirus expressing vesicular stomatitis virus (VSV) glycoprotein was harvested from culture media and concentrated by centrifugation (Sorvall SS-34 rotor) at 48,000*g* for 90 min at 4 °C. Pelleted virions were resuspended in Opti-MEM medium (Invitrogen) at 4 °C, after which functional virion titers were determined using target HT1080 human fibrosarcoma cells (Invitrogen) in the presence of the antibiotic blasticidin (Invitrogen). To generate a stable HT1080 cell line expressing luciferase, HT1080 cells were transduced with lentivirus virions (titer, 10^7 transducing units per ml) in the presence of polybrene (Sigma), and blasticidin-resistant cells, expressing luciferase, selected by culturing the cells in the presence of blasticidin for 14 days. Stable cells were isolated and cryopreserved and/or expanded for use as target, luciferase expressing HT1080 cells for the vector-based RNAi studies described here.

Luciferase expressing HT1080 cells were plated at a density of 5×10^5 cells/well in 6-well plates (Corning). Fifteen hours later, the cells were transfected with four or 10 μg of plasmid pXL-BacII_SmU6-shLuc or pXL-BacII_SmU6-shScramLuc or 10 pmol siLuc using the Lipofectamine 2000 transfection system (Invitrogen). A control group of cells exposed only to lipofectamine was included. One day later the cells were washed with PBS, after which plasmid transformed cells were incubated for 96 h in DMEM supplemented with 10% FBS, penicillin and streptomycin. Cells were counted and lysed in CCLR buffer (Promega) in order to determine levels of luciferase activity (see section 2.7).

2.6. Transfection of schistosomules with plasmids, siRNA and dsRNA

For the studies on RNAi in schistosomes, schistosomules were transfected with plasmid, dsRNA or siLuc by square wave electroporation. Specifically, schistosomules were transferred to cuvettes (4 mm gap, BTX, San Diego, CA, USA) containing media supplemented with 1 μg siLuc, 30 μg dsRNA or 20 μg of plasmid (pXL-BacII_SmU6-shLuc or pXL-BacII_SmU6-shScramLuc) and then subjected to a square wave pulse of 125 V of 20 ms duration, using a BTX ElectroSquarePoratorTM ECM830, as previously described (Kines et al., 2010). Subsequently, the transfected worms were transferred to pre-warmed Basch's medium and maintained in culture for 48 h until mRNA encoding firefly luciferase was introduced into the cultured worms by electroporation. After electroporation, the schistosomules were maintained in culture for 3 h and then washed free of culture media, and wet pellets of worms snap frozen and stored at -80 °C.

2.7. Luciferase activity assay

HT1080 cells and schistosomules were harvested, washed three times with schistosomule wash medium and stored as wet pellets at −80 °C. Pellets of schistosomules were subjected to sonication (3×5 s bursts, output cycle 4, Misonix Sonicator 3000, Newtown, CT 06470, USA) in 300 μl of 1x CCLR lysis buffer (Promega). Pellets of 1 x 10^6 HT1080 cells (cell numbers were determined by replicate counts of Trypan blue-stained cells using a hemacytometer) were lysed in 300 μl of 1x CCLR lysis buffer. The lysates were clarified by centrifugation in a microfuge at 4 °C, and analyzed for luciferase activity. Aliquots of 100 μl of clarified lysate were injected into 100 μl luciferin (Promega) at 25 °C, mixed and the relative light units (RLUs) determined in a tube luminometer (Sirius, Berthold, Pforzheim, Germany) 10 s later (Correnti and Pearce 2004; Kines et al., 2010). Duplicate samples were measured, with results presented as the average of the readings of RLU/s/mg of soluble schistosome protein or, for HT1080 cells, as RLU/s/10^3 cells. Recombinant firefly luciferase (Promega) was included as a positive control. The protein concentration in the soluble fraction of the schistosome extract was determined using the bicinchoninic acid assay (BCA kit, Pierce, Rockford, IL, USA).

2.8. Statistical analysis

Statistical comparisons were made by ANOVA. When significant differences were found among groups, a Student's *t*-test between controls and treatment conditions was applied, as appropriate. *P*-values of $\leqslant 0.05$ were considered to be significant and *P*-values of $\leqslant 0.01$ highly significant.

3. Results

3.1. Promoter region of the U6 non-coding RNA gene of S. mansoni

An identical match to the *S. mansoni* U6 gene was located in sequences in contig shisto3397e03.p1k of the draft genome assembly for *S. mansoni*. Flanking sequences upstream of the copy of the U6 gene in this contig were targeted for PCR (see section 2.2); ~474 bp upstream and flanking the U6 gene as well as part of the 5′-terminus of the U6 gene were amplified from genomic DNA of *S. mansoni*, cloned and sequenced (Fig. 1), therefore confirming the sequence identity as being identical to that in contig shisto3397e03.p1k. The 270 nt flanking and upstream of this copy of the *S. mansoni* U6 gene have been assigned GenBank accession number HQ540317.

The putative *S. mansoni* U6 promoter and U6 gene were aligned with those from human (GenBank X07425) and mouse (GenBank X06980). The schistosome U6 non-coding RNA gene sequence is 96% identical to its human and murine orthologues. The sequence of the putative promoter upstream of the *S. mansoni* U6 gene diverged substantially from the mammalian sequences. Notwithstanding, we attempted to locate motifs characteristic of U6 gene promoters. The enhancer region, also known as the distal sequence element (DSE), consists of an octamer motif (OCT) and a *Sph*I post-octamer homology (SPH) element. The core region comprises a proximal sequence element (PSE) and a TATA-like element (Dahlberg and Schenborn, 1988; Sturm et al., 1988; Schaub et al., 1999). Based on greater or lesser identity to the human and mouse motifs, we identified these prospective motifs of the enhancer and core regions in the 270 bp flanking the schistosome U6 gene; SPH element (21 residues), -258 ATTTGGTTGTTTAAAAATATA -238; OCT (eight residues), -230 ATTACTAT -223; PSE (21 residues), -71 GTGCAAATGAGTGAATGAACG -51; and the TATA box (seven residues), -31 TATGATA -25. These motifs are annotated on the multiple sequence alignment of the U6 genes in Fig. 1.

3.2. U6 promoter is active in vector-based RNAi

We constructed plasmids pXL-BacII_SmU6-shLuc and pXL-BacII_SmU6-shScramLuc (Fig. 2A). The short hairpin predicted to be

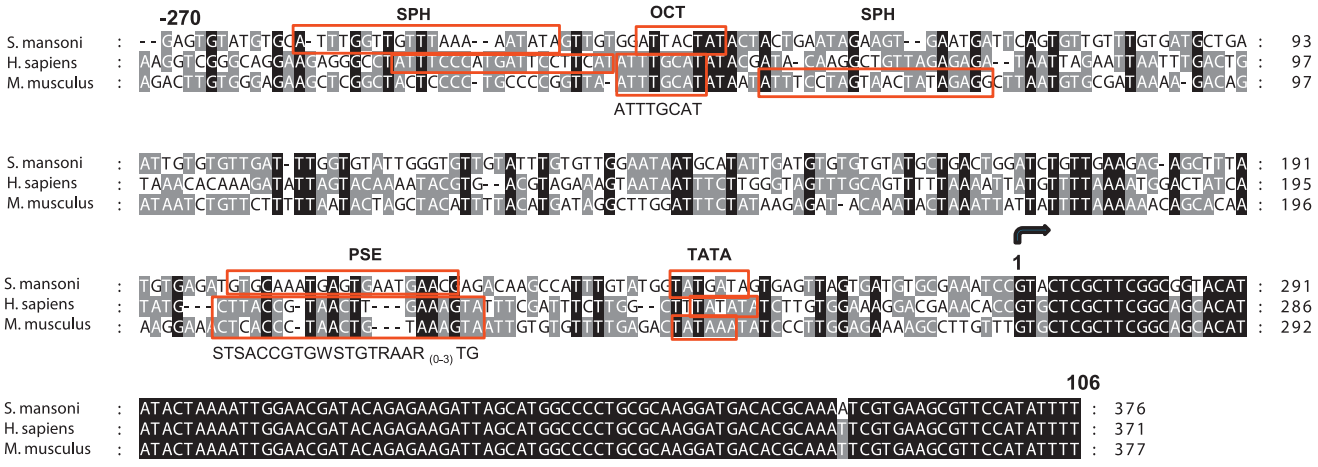

Fig. 1. Multiple sequence alignment of the U6 genes of *Schistosoma mansoni*, *Homo sapiens* and *Mus musculus*. The multiple alignment was assembled with ClustalW (Thompson et al., 1994) using BioEdit, version 7.0.5 (Hall, 1999) and the box shade feature of GeneDoc and the following sequences: *S. mansoni* (GenBank L25920 (coding region) and HQ540317 (upstream flanking region)); human, (GenBank X07425), mouse (GenBank X06980). Residue one (black, curved arrow) is the first nucleotide (nt) of the U6 RNA gene sequence. Characteristic motifs of the enhancer and core regions of the U6 gene promoter are identified or predicted (red colored rectangles). The enhancer distal sequence element includes an octamer motif (OCT) and *Sph*I post-octamer homology (SPH) element and the core region comprises a proximal sequence element (PSE) and a TATA-like element. Consensus sequences are indicated under the PSE and OCT motifs. Note that the relative positions for the SPH and OCT elements are reversed in mouse U6.

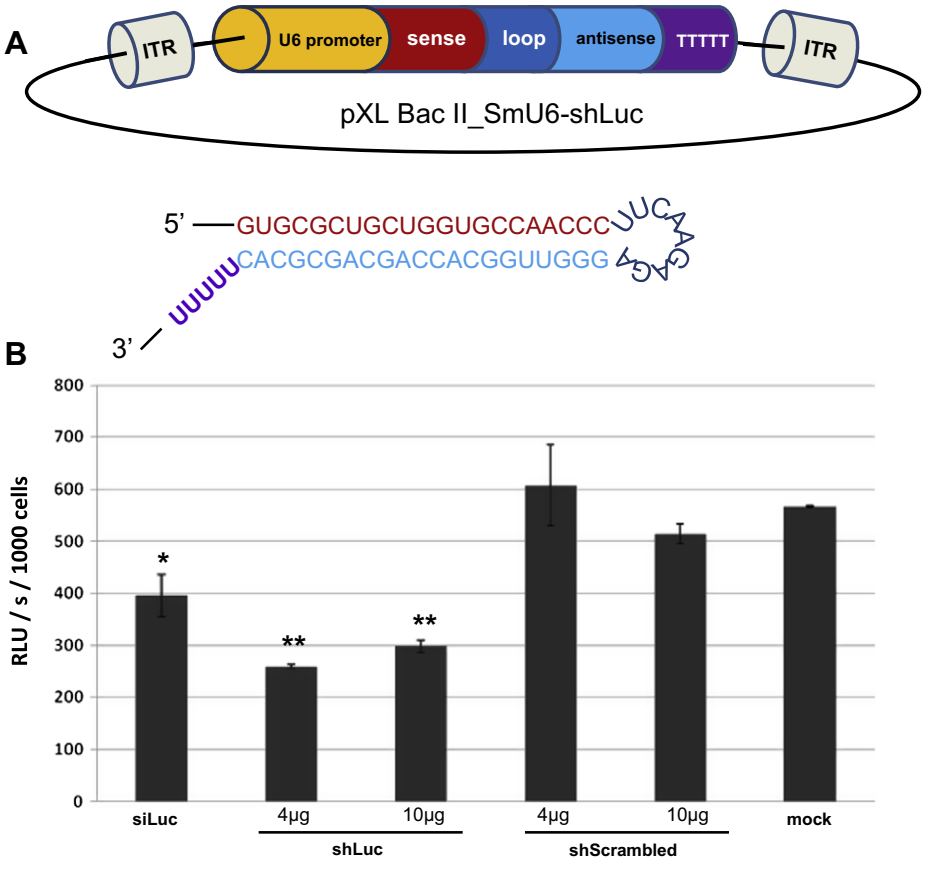

Fig. 2. Episomal vector-based RNA interference (RNAi) of firefly luciferase activity in human fibrosarcoma HT1080 cells. A) Schematic illustration of the insert of plasmid of pXL-BacII_SmU6-shLuc, including the inverted terminal repeats (ITR) flanking the short hairpin (sh)RNA cassette, the *Schistosoma mansoni* U6 gene promoter, the sense strand (21 nucleotide (nt), residues 851–871 of the gene encoding firefly luciferase, the nine residue loop, the 21 nt anti-sense strand and the TTTTTT terminator residues. The predicted shRNA is shown below the plasmid construct. B) Luciferase activity in HT1080 cells expressed as relative light units (RLU)/s/10^3 cells. HT1080 cells were transfected with 1 µg of small interfering RNA of 21 nt in length, specific for residues 851–871 of firefly luciferase (siLuc) ; 4 µg pXL-BacII_SmU6-shLuc (shLuc_4 µg); 10 µg of pXL-BacII_SmU6-shLuc (shLuc_4 µg); 4 µg plasmid pXL-BacII_SmU6-shScramLuc (shScrambled_4 µg); 10 µg of control plasmid pXL-BacII_SmU6-shScramLuc (shScrambled_10 µg); and no treatment control (mock). Bars are ± S.D. ($n = 2$). Significant differences between treated groups and the (mock) control are indicated: ∗, $P \leqslant 0.05$; ∗∗, $P \leqslant 0.01$.

transcribed from the U6 promoter of this episome includes the 21 nt sense strand specific for residues 851–871 of the luciferase transcript, a loop of nine residues, the 21 residue antisense strand, and the UUUUU termination signal. The control construct pXL-BacII_SmU6-shScramLuc includes the same residues in the target strand, but the order of the residues was scrambled. First, we transfected HT1080 cells with the vector based RNAi constructs. A HT1080 cell line that stably expressed firefly luciferase was used for this experiment, with the aim of vector-based RNAi targeting the 'endogenous' luciferase. At 96 h after transfection with the vector RNAi plasmids or with siRNA, statistically significant knockdown of luciferase activity was evident. In particular, luciferase activity was reduced by 54.3% and 47.5% in HT1080 cells transfected with 4 or 10 μg of pXL-BacII_SmU6-shLuc, respectively, compared with untreated, control HT1080 cells (both $P \leqslant 0.01$), and by 30.2% in cells exposed to the siRNA transfected cells ($P \leqslant 0.05$). By contrast, no reduction in luciferase activity was evident in cells transfected with the control construct, pXL-BacII_SmU6-shScramLuc (Fig. 2B). Similar findings were observed in each of two biological replicates of this experiment. These results indicated that the putative *S. mansoni* U6 promoter drives short hairpin expression in a human cell line against an endogenously expressed reporter gene.

3.3. Schistosome U6 promoter-driven shRNA induces RNAi in schistosomules

The activity of pXL-BacII_SmU6-shLuc was examined in cultured schistosomules. At 48 h after transfection with the vector RNAi plasmid, or with dsRNA or siRNA, statistically significant knockdown of luciferase activity was evident. Specifically, luciferase activity was reduced by 47.5% in schistosomules transfected with 20 μg of pXL-BacII_SmU6-shLuc compared with schistosomules treated with 20 μg of the control plasmid, pXL-BacII_SmU6-shScramLuc (183,030.5 versus 348,651.3 RLU/s/mg) ($P \leqslant 0.05$) (Fig. 3). The RNAi induced with dsRNA or siRNA was even greater than that with pXL-BacII_SmU6-shLuc, both > 95% knockdown of luciferase activity compared with the worms transfected with the control plasmid, pXL-BacII_SmU6-shScramLuc ($P \leqslant 0.01$). More luciferase activity was seen in schistosomules not transfected with any nucleic acids ($P \leqslant 0.01$), a control group that was termed 'mock' (Fig. 3). Similar findings were observed in three of four replicates of the experiment.

4. Discussion

RNAi is a widely employed approach in reverse genetics for specific knockdown of target genes. The RNAi mechanism is conserved among eukaryotes, including schistosomes (see Krautz-Peterson et al., 2010). RNAi is triggered by dsRNA and results in inactivation of the target gene through degradation of homologous mRNAs and/or inhibition of mRNA translation. In the context of vector-based RNAi, the molecular biology of RNAi has been reviewed (e.g., Cheng and Chang, 2007; Sliva and Schnierle, 2010). In brief, episomal or integrated transgenes, comprising pol III promoters driving target sequence-specific cassettes, lead to over-expression of shRNAs, of ~21 nt of stem (range 19–29 nt) and ~9 nt (range 4–23) of loop sequences (Brummelkamp et al., 2002; Miyagishi and Taira, 2002; Paddison et al., 2002). After export from the nucleus the shRNA is cleaved by Dicer and enters the RNA-induced silencing complex, which unwinds the RNA and targets the specific mRNA for degradation. Vector-based RNAi provides advantages over the direct introduction of siRNA or dsRNA to target cells, including long-term and/or conditional gene silencing. It also can be cost effective, given that synthesis of siRNA is expensive, its effect transient and in addition, long dsRNA can induce a cytotoxic response in mammalian cells (Williams, 1999). On the other hand, the RNAi effect using a vector-based system is more stable due to sustained production of shRNA.

We isolated 270 bp of genomic DNA flanking the *S. mansoni* U6 small nRNA gene, a component of the spliceosome machinery (see Copeland et al., 2009), and constructed a RNAi expression cassette using this putative promoter to drive expression of shRNAs specific for firefly luciferase. We observed significant knockdown of luciferase in human fibrosarcoma cells and in schistosomules transfected with the expression cassette. The findings demonstrated that the *S. mansoni* U6 gene promoter drove shRNA expression that induced RNAi in a human cell and in schistosomules. In addition to schistosome and human U6 gene promoters, orthologous U6 promoters from pathogenic amebae, *Drosophila*, sea squirts, fugu fish, chicken and bovines also function in Pol III driven vector-based RNAi expression systems (Lambeth et al., 2005; Wakiyama et al., 2005;

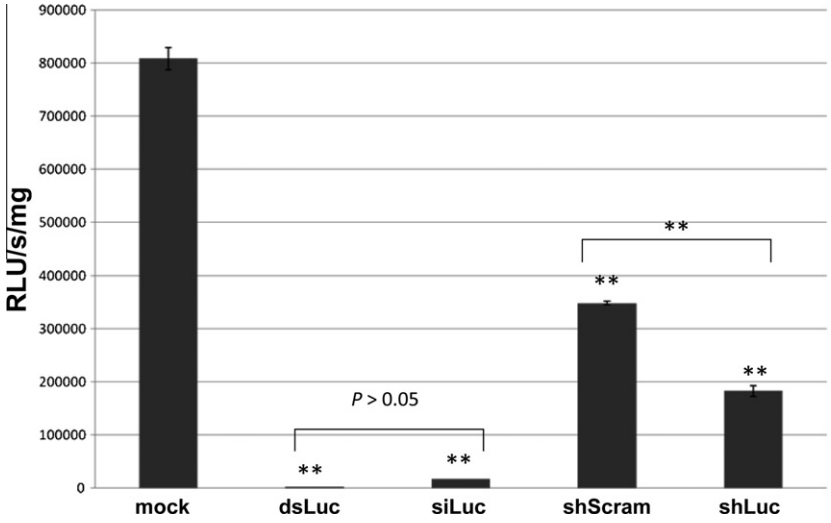

Fig. 3. Episomal vector-based RNA interference (RNAi) of firefly luciferase activity in 1 day old schistosomules. Luciferase activity in schistosomules expressed as relative light units (RLU)/s/mg of protein. One day old schistosomules were electroporated in the absence of plasmid or RNAs (mock), 30 μg of firefly luciferase double stranded RNA (dsLuc), 10 μg of small interfering RNA of 21 nt in length, specific for residues 851–871 of firefly luciferase (siLuc), 20 μg of control plasmid pXL-BacII_SmU6-shScramLuc (ShScram) and 20 μg of plasmid pXL-BacII_SmU6-shLuc (shLuc). Bars are ± S.D. ($n = 2$). Significant differences between treated groups and the (mock) control are indicated: *, $P \leqslant 0.05$; **, $P \leqslant 0.01$.

Wise et al., 2007; Nishiyama and Fujiwara, 2008; Zenke and Kim, 2008; Linford et al., 2009).

Although this *S. mansoni* U6 gene promoter is active in schistosomes and in HT1080 cells, the knockdown experiments performed in HT1080 cells were more consistent and reproducible than those with schistosomules. However, even in HT1080 cells we did not observe a concentration effect; 10 μg did not induce significantly greater silencing than 4 μg of pXL-BacII_SmU6 shLuc. This may reflect saturation of the cellular microRNA/shRNA pathway leading to competition for limited cellular factors such as nuclear karyopherin exportin-5 (see Grimm et al., 2006; Snøve and Rossi, 2006). The non-specific reduction of luciferase activity in schistosomules detected in the control shRNA group may reflect dysfunction of the mRNA translation machinery. Schistosomules clearly are less tractable for these approaches than mammalian cell lines but since functional genomics tools are needed for schistosomes, and because cell lines are not available for schistosomes, we have targeted larval schistosomes here to investigate a prospective new approach (Mann et al., 2008, 2010). Cultured HT1080 cells not only have a larger surface area, but here were transfected only once with the vector short hairpin plasmid whereas the schistosomules were transfected with this plasmid and, secondly, with the mRNA encoding luciferase. Exposing worms twice to electroporation may be deleterious and reflected in inconsistent outcomes in the replicates. Moreover, knockdown of luciferase in these particular HT1080 cells represented silencing of an 'endogenous' gene since the cells expressed firefly luciferase from integrated lentiviral transgenes. Perhaps most critical was that since schistosomules are multicellular, some but not all of the cells were transformed with both pXL-BacII_SmU6-shLuc and luciferase mRNA. Cells of the digestive tract and/or tegument are likely transduced by the plasmid and/or the mRNA, whereas more internal cells may not have been transfected. Nonetheless, we can predict that after entry into the schistosome cell, the episome was transcribed and processed, and the siRNAs transmitted to adjacent cells by transporters such as Sid 1 (Krautz-Peterson and Skelly, 2008). In this regard and to improve assay reproducibility, increasing the time between the plasmid and the luciferase mRNA transfection might be beneficial so that siRNAs might spread from the transfected cells, leading to enhanced knockdown.

Recently, proof-of-principle for vector-based RNAi was demonstrated in *S. mansoni*, using a MLV (retrovirus) vector (Tchoubrieva et al., 2010). However, those investigators used a RNA polymerase II dependent promoter and a long hairpin of ~120 bp targeting a protease involved in hemoglobinolysis. Whereas this retroviral vector-based RNAi system is functional, there may be advantages to deploying a Pol III dependent promoter: the U6 promoter is known to competently and continuously transcribe small RNAs, is highly active in all or most cells and tissues, and its diminutive size fits within the limits on cargo size of the expression cassette (Paddison et al., 2002; Scherr and Eder, 2007). The significance of both findings with vector-based RNAi in schistosomes, the present findings and those of Tchoubrieva et al. (2010) is that vector-based RNAi should allow targeting of any schistosome gene for continuous knockdown and subsequent examination of the importance of the targeted gene. Moreover, plasmid pXL-BacII, from which we constructed pXL-BacII_SmU6-shLuc, contains the inverted terminal repeats of transposon piggyBac (Li et al., 2005). Given that piggyBac is transpositionally active in *S. mansoni* (Morales et al., 2007), pXL-BacII_SmU6-shLuc could be utilized to generate transgenic schistosomes, that inherit trans-generational silencing of report luciferase, by co-transfecting schistosomes with this shRNA inducing plasmid along with the piggyBac transposase.

High throughput RNAi analysis will advance functional genomics for schistosomes, and indeed parasitic helminths at large. We anticipate that the information presented here describing the schistosome U6 promoter and its activity in vector-based RNAi will contribute to establishment of these analyses. We are interested to establish long-term, stable and trans-generational RNAi to aid discovery of essential schistosome genes which could, in turn, be targeted in new approaches for treatment and control of schistosomiasis.

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ORIGINAL PAPER

Human U6 promoter drives stronger shRNA activity than its schistosome orthologue in *Schistosoma mansoni* and human fibrosarcoma cells

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Abstract Blood flukes or schistosomes are the causative agents of human schistosomiasis, one of the major neglected tropical diseases. Draft genome sequences have been reported for schistosomes, but functional genomics tools are needed to investigate the role and essentiality of the newly reported genes. Vector based RNA interference can contribute to functional genomics analysis for schistosomes. Using mRNA encoding reporter firefly luciferase as a model target, we compared the performance of a schistosome and a human promoter from the U6 gene in driving shRNA in human fibrosarcoma cells and in cultured schistosomes. Further, both a retroviral [Murine leukemia virus (MLV)] and plasmid (*piggyBac*, pXL-Bac II) vector were utilized. The schistosome U6 gene promoter was 270 bp in length, the human U6 gene promoter was 264 bp; they shared 41% identity. Following transduction of both HT1080 fibrosarcoma cells and schistosomules of *Schistosoma mansoni* with pseudotyped MLV virions, stronger knockdown of luciferase activity was seen with the virions encoding the human U6 promoter driven shRNA than the schistosome U6 promoter. A similar trend was seen after transfection of HT1080 cells and schistosomules with the pXL-Bac-II constructs—stronger knockdown of luciferase activity was seen with constructs encoding the human compared to schistosome U6 promoter. The findings indicate that a human U6 gene promoter drives stronger shRNA activity than its schistosome

Raphaël Duvoisin and Mary A. Ayuk contributed equally.

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orthologue, not only in a human cancer cell line but also in larval schistosomes. This RNA polymerase III promoter represents a potentially valuable component for vector based RNA interference studies in schistosomes and related platyhelminth parasites.

Keywords Schistosome · Schistosoma mansoni · Short hairpin RNA · U6 gene · Promoter · Firefly luciferase · RNA interference · Murine leukemia virus · *piggyBac* transposon

Introduction

Vector-based RNAi approaches that lead to chromosomal integration of transgenes encoding cassettes expressing small interfering RNAs (siRNAs) can circumvent deficiencies with cytoplasmic RNAi approaches by providing long-term, continuous and/or conditional gene silencing (ter Brake et al. 2006; Moore et al. 2010; Sliva and Schnierle 2010). By contrast, delivery of dsRNAs/siRNAs from the exterior of the cell to the cytoplasm frequently delivers only transitory gene silencing, may be inaccessible to some developmental stages and/or tissues, can be expensive and, in addition, cytoplasmic dsRNAs may induce apoptosis and interferon signaling responses in some situations (Haasnoot et al. 2007; Sliva and Schnierle 2010; Handa et al. 2011).

The schistosomes are considered the most important of the human helminth parasites in terms of the morbidity and mortality which they cause (Hotez et al. 2008; Han et al. 2009; Rollinson 2009; Gurarie et al. 2011). In addition, *Schistosoma haematobium* is a cause of squamous cell carcinoma of the bladder and is classified as a Group 1 carcinogen by the International Agency for Research on Cancer (Bouvard et al. 2009). Following the recent reports of the draft genome sequences of two of the major human schistosome species (Berriman et al. 2009; *Schistosoma japonicum* Genome Sequence and Functional Analysis Consortium 2009), efforts have increased towards harnessing the functional genomics approaches widely used in model species and human gene therapy for investigation of the importance and essentiality of these new schistosome genes (Stefanic et al. 2010; Collins et al. 2011). Information obtained with these advances can be expected to guide development of new interventions for these major neglected tropical disease pathogens (Brindley et al. 2009; Han et al. 2009).

In relation to this interest in expanding functional genomics for schistosomes, it has been demonstrated that both pseudotyped murine leukemia virus (MLV) and the transposon *piggyBac* can transduce developmental stages of schistosomes, leading to chromosomal integration of retroviral transgenes and transgene reporter activity (Morales et al. 2007; Kines et al. 2008; Yang et al. 2010; Rinaldi et al. 2011). Moreover, vector-based RNAi approaches have been reported in schistosomes (e.g. Zhao et al. 2008). The goal of the present investigation was to compare and contrast the performance of two U6 gene promoters, one from *S. mansoni*, the other from the human genome, in driving short hairpin RNAs in cultured schistosomes. There may be advantages to deploying a RNA polymerase (Pol III) promoter: the U6 promoter is known to competently and continuously transcribe small RNAs, is highly active in all or most cells and tissues where this has been examined (information on this aspect is not available for schistosomes, so it is not known whether Pol III is likewise widely active in cells of this flatworm), and its diminutive size fits within the limits on cargo size of the expression cassette (Paddison et al. 2002; Scherr and Eder 2007). Defining active promoter elements for vector based RNAi in schistosomes can be expected to be worthwhile since studies of the human schistosomes are more problematic than established approaches with cultured mammalian cells or with more well studied model species such as *Caenorhabditis elegans* and *Drosophila melanogaster*. The present findings revealed the superior performance of a human U6 promoter when compared to an endogenous U6 promoter from *S. mansoni* in driving active shRNAs in cultured schistosomes.

Materials and methods

HT1080 fibrosarcoma cells expressing firefly luciferase

We employed a genetically modified HT1080 human fibrosarcoma cell line, that constitutively expresses luciferase, in these studies of short hairpin RNA (Ayuk et al. 2011). In brief to generate a HT1080 cell

line stably expressing luciferase, HT1080 cells (ATCC, Manassas, VA) were transduced with lentivirus virions (ViraPower Promoterless Lentiviral Gateway Expression System, Invitrogen) that includes a gene conferring resistance to blasticidin, and the firefly luciferase gene driven by the *S. mansoni* spliced leader RNA gene promoter (see Kines et al. 2006) in the presence of polybrene (Sigma). Blasticidin-resistant cells expressing luciferase were selected by culturing in the presence of blasticidin for 14 days. Stable cells were isolated, cryopreserved and/or expanded for use as target, luciferase expressing HT1080 cells for the vector based RNAi (below).

Schistosoma mansoni

Biomphalaria glabrata snails infected with the NMRI (Puerto Rican) strain of *S. mansoni* were supplied by Dr. Fred Lewis, Biomedical Research Institute, Rockville, MD, USA. Cercariae released from infected *B. glabrata* snails were mechanically transformed into schistosomules. In brief, cercariae were concentrated by centrifugation (425 g/10 min) and washed once with schistosomule wash medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 200 units/ml of penicillin, 200 µg/ml of streptomycin, 500 ng/ml of amphotericin B and 10 mM HEPES). Cercarial tails were sheared from the larvae by 20 passes through 22 gauge emulsifying needles after which schistosomule bodies were isolated from tails by Percoll gradient centrifugation (Lazdins et al. 1982). Schistosomula were washed three times in schistosomule wash medium and cultured in Basch's medium (Basch 1981) at 37°C under 5% CO_2 in air for up to 14 days. Some cultures were supplemented with washed human erythrocytes, at a density of 1 µl of packed red cells per ml of medium (Mann et al. 2010).

Construction of shRNA expression cassettes targeting firefly luciferase

The sequence of the human U6 small nuclear RNA gene (GenBank X07425) was used to design primers HsU6F and HsU6R (Table S1) and the primers employed to amplify upstream and part of the 5′-terminus of the U6 gene from genomic DNA from human white blood cells using a kit (ENZA, Omega Bio-tek, Doraville, GA). The amplicon was ligated into pCR4 TOPO (Invitrogen), the insert sequenced, the nucleotide sequence aligned with other human and schistosome U6 promoters, and this human U6 promoter sequence included in the shRNA expression cassette in the MLV pLNHXΔD70 (generated by the removal of *Drosophila* heat shock 70 gene promoter from pLNHX using *Xho* I) (Suttiprapa, unpublished) and *piggyBac* (pXL-BacII) constructs assembled here (Fig. 1). The *S. mansoni* U6 promoter has been described (Ayuk et al. 2011).

A two-step PCR approach was performed to construct the shRNA expression cassettes targeting firefly luciferase (Gou et al. 2003; Linford et al. 2009; Ayuk et al. 2011) that included either a human or a schistosome U6 promoter. The approach involved two rounds of PCR using one universal primer, specific for the promoter (human or schistosome) and two unique target sequences, the first oligonucleotide to add the sense strand of the shRNA, the second to add the loop region, anti-sense strand and terminal thymidines. For the hairpin sense strand, we included 21 residues, nucleotides 851-871, 5′-GTGCGCTGCTGGTGCCA ACCC-3′, of the firefly luciferase (cDNA) gene in pGL3 (Promega) because this site and target sequence length have been demonstrated in shRNA studies to lead to complete or near complete knockdown of luciferase activity in mammalian and insect cell lines (Gou et al. 2003; Wakiyama et al. 2005). For a control shRNA, we constructed cassettes where the 21 target residues 851-871 of the luciferase gene were scrambled, as follows, 5′-ACCTACTGGGCAGGGAGCC GC-3′, using the same forward primer (above) and the reverse primers (Table S1). The final products were sized by agarose gel electrophoresis, eluted from gel, cleaved with *Xho* I for pLNHX and *Bam*H I and *Xho* I for pXL-Bac II (Li et al. 2005), and ligated into linearized retroviral or transposon vector plasmids. Figure 1 provides an illustration of the constructs, termed pLNHX_SmU6_shLuc (GenBank accession JN255692), pLNHX_HsU6_shLuc (JN255690), pLNHX_HsU6_ScramLuc (JN255691), pXL-Bac-II_SmU6-shLuc (HQ677838), pXL-BacII_SmU6-shScramLuc (HQ677839), pXL-BacII_HsU6-shLuc (JF298906), and pXL-BacII_HsU6-shScramLuc (JF298907). Sequences of oligonucleotide primers used in assembly of these shRNA expression cassettes are provided in Table S1.

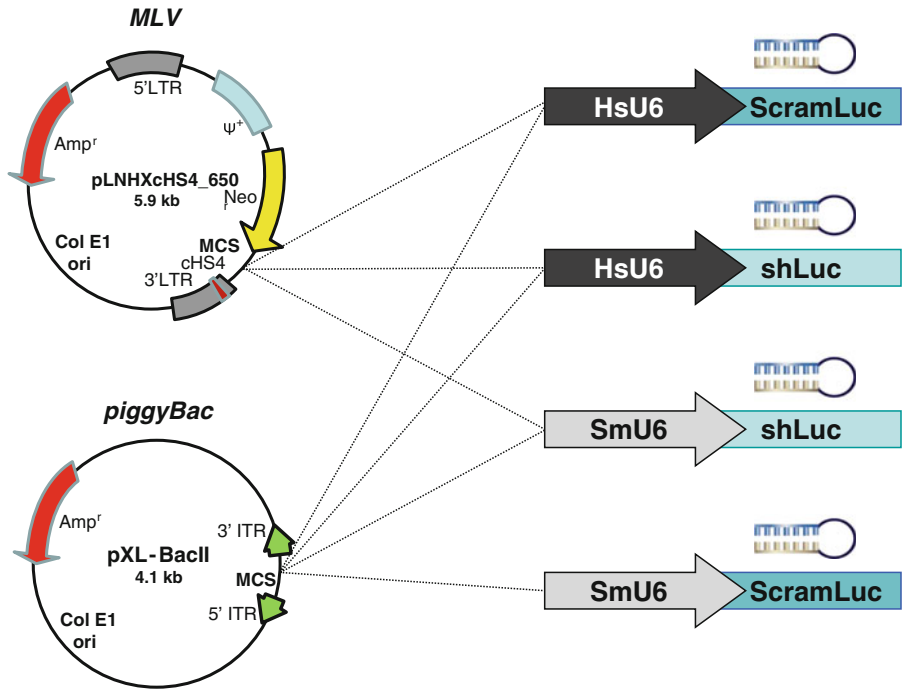

Fig. 1 Schematic illustration of the retroviral pLNHX (murine leukemia virus, MLV) and pXL-Bac II (transposon *piggyBac*) based plasmids (*left*) and shRNA expression cassettes (*right*) modified here by insertion of shRNA expression cassettes. The shRNA expression cassettes target firefly luciferase or a 'scrambled' control sequence. *MCS* Multiple cloning site, *ITR* inverted terminal repeat (regions of the transposon), *LTR* long terminal repeat (of the retrovirus), *cHS4* chicken DNase-I hypersensitive site 4 (a prototypic chromatin insulator)

Production of pseudotyped retroviral virions

Plasmid constructs, prepared with a maxiprep kit (5 Prime, Gaithersburg, MD), were employed to produce the virions as described (Kines et al. 2010; Rinaldi et al. 2011). In brief, GP2-293 cells were co-transfected with the MLV encoding plasmid (Fig. 1) along with pVSVG, which encodes vesicular stomatitis virus glycoprotein (the pseudotyping envelope) using liposomes (Lipofectamine 2000, Invitrogen). Culture supernatants were collected 48 h later, filtered through 0.45 µm pore size membranes, the filtrate incubated with DNAse I (New England Biolabs, Ipswich, MA) to remove contaminating plasmids, and virions in culture supernatants recovered by centrifugation (Sorvall SS34 rotor, 50,000*g*, 90 min, 4°C). Pelleted virions were resuspended in schistosomule wash medium (above), virion titers estimated using quantitative PCR (Mann et al. 2011), and aliquots of virions stored at −80°C until needed. pLNHXΔD70 was employed to produce control virions without shRNA encoding cassettes.

Messenger RNA encoding firefly luciferase

For preparation of firefly luciferase mRNAs (mLuc), in vitro transcriptions of capped RNAs from template PCR products from the luciferase gene in pGL3-Basic (Promega) were accomplished using the mMessage mMachine T7 Ultra kit (Ambion) (Correnti and Pearce 2004). mLuc was precipitated with one volume of 5 M ammonium acetate and 2.5 volumes of 95% ethanol after which the precipitate was dissolved in water and concentration and purity determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Transduction of HT1080 cells

Luciferase expressing HT1080 cells were plated at a density of 2 to 5 × 10^5 in 6-well (35 mm) plates to achieve 30–50% confluence the following day. For transduction with retroviral virions, pLNHX_HsU6_shLuc or pLNHX_HsU6_Scram control virions (6.5 × 10^8 virions) were added to the cells which

were then cultured for 18 h in 2 ml of DMEM supplemented with 10% FBS, 100 units/ml of penicillin, 100 μg/ml of streptomycin and 8 μg/ml polybrene. Culture media were changed the following day. The transduced cells were harvested at 96 h after addition of virions. For some experiments, transduced HT1080 cells were subjected to selection on G418 at 500 μg/ml for 10 days, since the retroviral encodes the neomycin resistance gene (Fig. 1), with changes of culture media every second day. After harvesting, cells were counted and lysed in CCLR buffer (Promega) in order to determine levels of luciferase activity (below).

In like fashion, HT1080 cells at ~90% confluence were transfected with 4 μg of plasmid pXL-BacII_SmU6-shLuc, pXL-BacII_SmU6-shScramLuc, pXL-BacII_HsU6-shLuc or pXL-BacII_HsU6-shScramLuc using Lipofectamine 2000. A control group of cells not transfected with plasmids but exposed to the liposomes was included (mock). The following day, cells were washed with 1× PBS, and then cultured for 96 h in DMEM supplemented with 10% FBS, 100 units/ml of penicillin, 100 μg/ml of streptomycin. Cells were counted and lysed, as above.

Transduction of schistosomules

For transduction of schistosomes, virions—at titers ranging from 2.6×10^9 to 1.5×10^{10} per treatment well—were added to cultures of 7 day old schistosomules, 10^3–10^4 schistosomules per well (24-well plates; Corning) in one ml schistosomule media containing 8 μg/ml polybrene. Media were changed at 18 h after addition of virions, after which schistosomules were maintained in culture for 3 days. At this point these schistosomules were transfected with mRNA encoding firefly luciferase. Three hours after electroporation the parasites were harvested for luciferase activity assays. In some experiments, the schistosomules were exposed to the virus by centrifugation (800*g*, 60 min, 21°C) in the presence of polybrene.

Schistosomules were transfected with pXL-Bac II constructs by square wave electroporation; the worms were transferred to cuvettes (4 mm gap, BTX, San Diego, CA, USA) containing media supplemented with 20 μg of plasmid (pXL-BacII_ SmU6-shLuc or pXL-BacII_SmU6-shScramLuc or pXL-BacII_ HsU6-shLuc or pXL-BacII_HsU6-shScramLuc) and then subjected to a square wave pulse of 125 V of 20 ms duration, using a BTX ElectroSquarePoratorTM ECM830, as described (Kines et al. 2010). Transfected worms were transferred to pre-warmed Basch's medium and maintained in culture for 48 h. mRNA encoding firefly luciferase was introduced into the cultured worms by electroporation (using the same conditions as for the plasmids). After electroporation, the schistosomules were maintained in culture for 3 h and then washed free of culture media, and wet pellets of worms snap frozen and stored at −80°C. [It is pertinent to note that exposing worms twice to electroporation may be deleterious, and reflected in inconsistent outcomes in the replicates (Ayuk et al. 2011)].

Luciferase activity assay

HT1080 cells and schistosomules were harvested, lysed, clarified supernatant (after centrifugation of lysates, 10,000*g*, 10 min 4°C), analyzed for luciferase activity. Aliquots of 100 μl of lysate were injected into100 μl luciferin (Promega) at 25°C, mixed, and the relative light units (RLUs) determined in a tube luminometer (Sirius, Berthold, Pforzheim, Germany) 10 s later (Correnti and Pearce 2004). Replicate samples were measured, with results presented as the average of the readings of relative light units (RLU) per second per μg of soluble HT1080 or schistosome protein. Recombinant firefly luciferase (Promega) was included as a positive control in order to verify that the luciferin substrate and luminometer were functioning in a normal, expected fashion. Protein concentration of soluble fractions of transduced HT1080 cell and schistosome extracts were determined using the bicinchoninic acid assay (BCA kit, Pierce, Rockford, IL).

Results

U6 promoters diverge between schistosomes and humans

Whereas it was clear from a recent study that a U6 promoter from *S. mansoni* drove shRNA expression (Ayuk et al. 2011), here we compared the performance of a human U6 promoter with a schistosome

orthologue since the human U6 promoter is used widely in shRNA studies (Makinen et al. 2006; Roelz et al. 2010), including its inclusion as the promoter in the commercial vector pENTR/U6 from Invitrogen. Several U6 genes from the human genome are active although it appears that the promoter termed U6-1 is the widely utilized variant (Kunkel and Pederson 1988; Domitrovich and Kunkel 2003) (GenBank X07425). We aligned the X07425 sequence, the human U6 promoter from pENTR/U6 (here termed Invitrogen_U6), and GenBank JN255693, a variant of the human U6 promoter we cloned and sequenced from the blood donated by a co-author of this report, and a *S. mansoni* U6 gene promoter (HQ540317) (alignment not shown but available from corresponding author). The schistosome U6 gene promoter was 270 bp in length, the human U6 gene promoter was 264 bp, and they shared 41% identity. By contrast, the three human orthologues aligned were nearly identical; X07425 differed from JN255693 and Invitrogen_U6 by the inclusion of an additional residue, a thymidine (T) at position-98. This T occurred outside conserved Pol III binding domains, SPH, OCT, PSE and TATA, which were identical in the three human sequences. However, clear differences were apparent in all four domains between the schistosome (HQ540317) and human U6 promoters (Ayuk et al. 2011). The human U6 gene promoter in GenBank JN255693 was included as the promoter in shRNA expression cassettes constructed here (Fig. 1).

Human U6 promoter drives shRNA activity in schistosomes

At the outset, we examined the human U6 promoter in the context of transduction of schistosomules by pLNHX based virions. Human fibrosarcoma HT1080 cells, stably expressing luciferase, and 7 day old schistosomules were transduced with retroviruses encoding the shRNA targeting firefly luciferase or a scrambled sequence (control). As shown in Fig. 2, in both the HT1080 cells (panel A) and the schistosomes (panel B), which had been transfected with firefly luciferase encoding mRNA (mLuc), substantial knockdown of luciferase was apparent in the target HT1080 (47% knockdown) and schistosome parasites (81%) (Fig. 2, panel A (HT1080 cells), and panel B (schistosomules) when the effect of the pLNHX_HsU6-shLuc and pLNHX_HsU6_Scram virions was compared. Similar findings were seen when transduced HT1080 cells were selected on geneticin/G418 for 10 days (49%) (Figure S1, panel A). Also, similar results were seen with schistosomules at 10 days after transduction by virions (60%) and in schistosomules exposed to the virus by centrifugation (85%) (Figure S1, panels B and C, respectively).

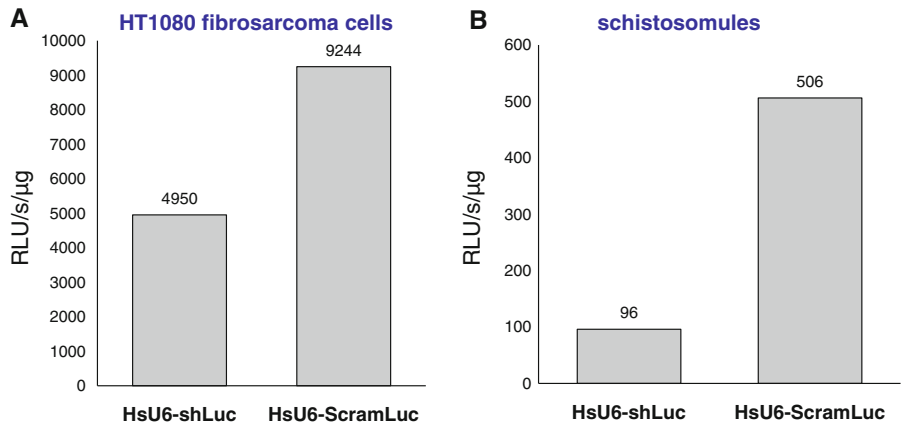

Fig. 2 Human U6 gene promoter driving short hairpin RNA (shRNA) in human HT1080 fibrosarcoma cells and 7 day old schistosomules of *Schistosoma mansoni* after transduction with MLV retroviral virions. **a** Knockdown of luciferase (47% knockdown) in HT1080 cells transduced with pLNHX_HsU6_shLuc (HsU6 shLuc) virions compared to control (HsU6 Scram) virions. **b** Knockdown (80%) of luciferase in schistosomules transduced with pLNHX_HsU6_shLuc (HsU6 shLuc) virions compared to control (HsU6 Scram) virions. Luciferase activity is expressed as relative light units per second per microgram of soluble protein (RLU/s/µg)

Following retroviral transduction, human U6 drives stronger shRNA than schistosome U6 in schistosomules

Having determined above that the heterologous human U6 promoter was active in shRNA cassettes in schistosomes, we next compared the performance of the schistosome and human U6 promoters in schistosomes. Groups of schistosomules were transduced with pLNHX_HsU6-shLuc, pLNHX_SmU6-shLuc virions, or control pLNHXΔD70 virions. An additional control group, the mock transduction group exposed only to polybrene, was included. In the experiment presented in Fig. 3, a knockdown of 38% luciferase activity was observed in schistosomules transduced with pLNHX_HsU6-shLuc virions compared to both mock and pLNHXΔD70 control groups. By contrast, no knockdown was observed in worms transduced with the pLNHX_SmU6-shLuc virions. This same trend was observed in two replicates of this experiment (not shown). Thus, the human U6 promoter drove stronger shRNA activity than its schistosome orthologue in *S. mansoni*. Indeed, the schistosome promoter was barely or not active under these conditions.

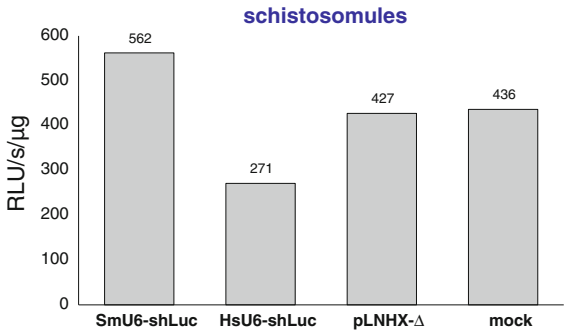

Fig. 3 Comparison between human U6 and schistosome U6 promoters driving short hairpin RNA targeting firefly luciferase in 7 day old schistosomules after transduction with MLV retroviral virions. Transduction with the human U6 promoter based pLNHX_HsU6_shLuc virions lead to knockdown (38%) whereas no knockdown was evident after transduction with the schistosome U6 promoter based pLNHX_SmU6_shLuc virions, when compared to the two control groups. These two control schistosomules were transduced with control MLV (pLNHXΔ) virions and not transduced with virions (mock) Luciferase activity is expressed as relative light units (RLU) per second per microgram of soluble protein (RLU/s/µg)

Human U6 promoter drove stronger shRNA activity than schistosome U6 promoter after delivery of transgenes in a transposon plasmid

In addition to MLV retroviral based transgenesis of schistosomes and HT1080 cells to compare the human and schistosome U6 promoters, we carried out parallel studies using the *piggyBac* transposon plasmid pXL-Bac II (Fig. 1). As above, we targeted HT1080 cells expressing luciferase and schistosomules transfected exogenously with luciferase encoding mRNA (mLuc). First, in each target type, the promoter driving the shRNA targeting luciferase produced a strong knockdown of luciferase in contrast to the control constructs targeting the scrambled sequence (Fig. 4, panels A and B). In both HT1080 and in schistosomules, the human U6 promoter delivered greater knockdown of luciferase activity than the schistosome U6 promoter; 88% versus 75% in HT1080, 80% versus 35% in schistosomules. By contrast, similar levels of luciferase activity were evident in cells (or schistosomes) transfected with the control constructs, pXL-BacII_SmU6-shScramLuc (SScram) or pXL-BacII_HsU6-shScramLuc (HScram). Similar findings were seen on each of two occasions these assays were performed (Figure S2). [Luciferase levels in the mock i.e. no shRNA control were somewhat higher than in cells (or schistosomules) transfected with the shRNA control plasmids, a phenomenon we have seen and discussed previously (Ayuk et al. 2011)].

Discussion

Deployment of shRNA for RNA interference represents a new strategy for functional genomics analyses of schistosomes and the human helminth parasites at large, which are major neglected disease pathogens. Whereas this is an emerging area, three recent reports have shown that both Pol II and Pol III promoters drive short hairpins in *Schistosoma japonicum* and *S. mansoni* that can silence target genes (Zhao et al. 2008; Tchoubrieva et al. 2010; Ayuk et al. 2011). The findings revealed that endogenous genes—*mago nashi* in *S. japonicum* and cathepsin B in *S. mansoni*, as well as reporter firefly luciferase, can be silenced following transfection of the schistosomes with transgene cassettes encoding cognate hairpin RNAs. Furthermore,

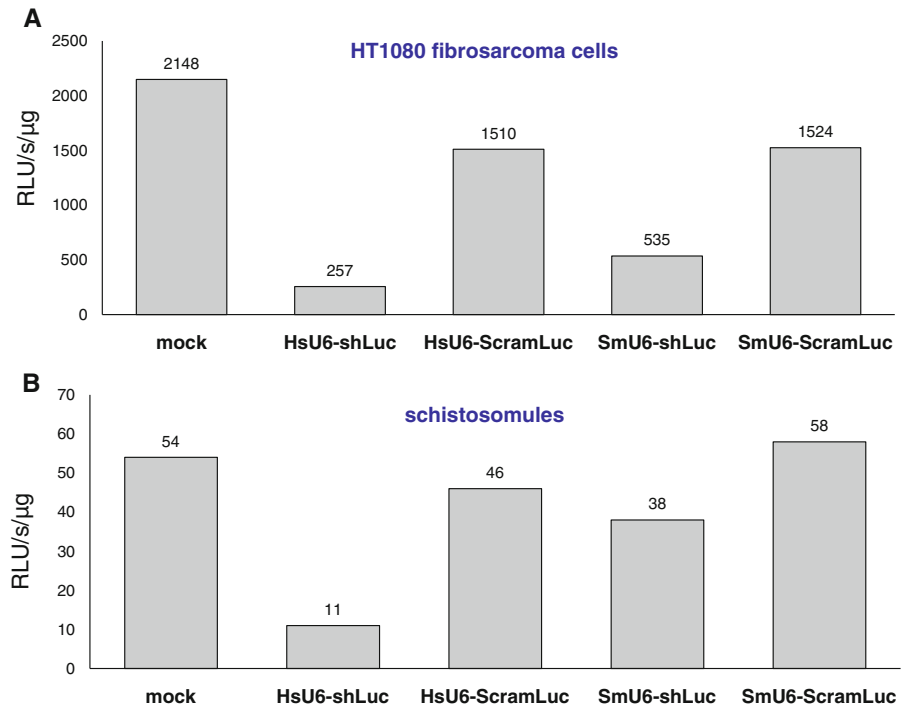

Fig. 4 Comparison between human U6 and schistosome U6 promoter driving short hairpin RNAs targeting firefly luciferase in a HT1080 cell line stably expressing firefly luciferase and in 14 day old schistosomules of *Schistosoma mansoni* after transfection with pXL-Bac II plasmids. **a** Strong knockdown was seen in cells transfected with both the human U6 (88% knockdown) and schistosome U6 (75%) promoters in comparison with mock-treatment control. By contrast, less knockdown was apparent in controls transfected with the human U6 and schistosome scrambled target sequence based plasmids, 30 and 29% respectively. **b** In schistosomules, stronger knockdown was seen with human U6 promoter (80% knockdown) compared with schistosome U6 promoter (30% knockdown) driving shRNA after transfection with pXL-Bac II plasmid constructs. Minimal or no knockdown was seen with the control plasmids, 15 and 0%. In both **a** and **b**, luciferase activity is expressed as relative light units per second per microgram of soluble protein (RLU/s/µg)

endogenous—*S. mansoni* actin (Tchoubrieva et al. 2010) and *S. mansoni* U6 (Ayuk et al. 2011)—and a heterologous promoter—human H1 (Zhao et al. 2008)—were shown to be active.

Here endo- and exogenous orthologues of U6 gene promoters were examined for performance in shRNA expression cassettes in *S. mansoni*. Promoters from the genomes of both *S. mansoni* and *Homo sapiens* were tested as components of the shRNA cassettes. The genomes of both humans and schistosomes include numerous copies of the U6 small nuclear RNA gene: there are at least nine copies of the human U6 gene, five of which are active (Domitrovich and Kunkel 2003), and nine to 55 copies of the *S. mansoni* orthologue (Copeland et al. 2009). The copy of the human U6 gene promoter tested here appeared to be identical or close to identical to U6-1 (Domitrovich and Kunkel 2003), a well-characterized copy that efficiently drives shRNAs in human cells and cells from non-congruent species (Boden et al. 2003; Roelz et al. 2010). We employed both pseudotyped retrovirus (MLV) and *piggyBac* based vectors, both known to integrate transgenes into chromosomes of this platyhelminth parasite (Morales et al. 2007; Kines et al. 2008). Notably, the findings revealed that the human U6 promoter displayed superior performance to the schistosome U6 orthologue: in both the retroviral and donor transposon contexts, the human U6 promoter drove stronger knockdown of firefly luciferase in a human fibrosarcoma cell line and in cultured schistosomes.

The promoters were of similar length, 264 nt for human U6 and 270 nt for *S. mansoni*, but were only 41% identical. The binding motifs for Pol III in the

human U6 promoter have been characterized (Domitrovich and Kunkel 2003). They include an enhancer region, also termed the distal sequence element (DSE). The DSE consists of an octamer motif (OCT) and a *Sph* I post-octamer homology (SPH) element. In addition, a core region comprises a proximal sequence element (PSE) and a TATA-like element (Dahlberg and Schenborn 1988; Sturm et al. 1988; Schaub et al. 1999; Myslinski et al. 2001). Based on greater or lesser identity to the human and mouse motifs, previously we identified these prospective motifs of the enhancer and core regions in the 270 bp upstream of a schistosome U6 gene (Ayuk et al. 2011). It is feasible that clear differences in sequence in the PSE and other domains—for example, TATGATA versus TATATA for the TATA motif; ATTACTAT versus ATTTGCAT for the OCT motif in the schistosome and human promoters, respectively (Ayuk et al. 2011)—were reflected in the differential activity of the human and schistosome U6 gene promoters. However, confirmation of the influence of these motif differences of the schistosome and human U6 promoters in schistosomes awaits further investigation. In addition, other copies of the *S. mansoni* U6 promoter may exhibit sequence differences that drive stronger shRNA expression, as has been demonstrated among the human U6 gene orthologues (Domitrovich and Kunkel 2003).

The present results extend earlier findings wherein it was demonstrated the schistosome U6 promoter drove shRNA in both targets—HT1080 fibrosarcoma cells and blood stage schistosomes. However, based on these findings, we recommend deployment of the human U6 promoter rather than the schistosome promoter in future shRNA investigations. Zhao et al. (2008) demonstrated the activity of the human H1 gene promoter for shRNAs in *S. japonicum*. Makinen et al. (2006) demonstrated that the human U6 was more efficient than the H1 promoter in silencing reporters in both human (293T) and murine (c166-GFP) cell lines, leading to 80% knockdown of the transcript encoding green fluorescent protein. Similar trends were apparent in vivo, in mouse brains. [H1 RNA is the RNA component of the human nuclear RNase P; the H1 promoter, which like that of U6 is a type 3 RNA polymerase III promoter, is unusually compact (Myslinski et al. 2001).] Moreover, shRNA expression from the human U6 promoter resulted in a four-fold increase in knockdown efficiency compared to expression from the mouse U6 promoter in human and in mouse cell lines (Roelz et al. 2010). Accordingly, the human U6 promoter appears in general to drive strong shRNA based knockdown; the present findings demonstrated that this also occurs in schistosomes. In less well studied helminth parasites (Rinaldi et al. 2008), it is reasonable to expect the human U6 gene would also be serviceable for shRNA investigations. Indeed, deploying the human U6 promoter could provide an initial way forward with functional studies in less well studied species, for example the helminth parasites of humanity at large, until endogenous (congruent) U6 genes could be isolated and tested. In relation to *S. mansoni* or other schistosome species, deploying the human U6 gene promoter should allow studies to progress while other copies of the schistosome U6 gene can be investigated.

To conclude, these findings indicated superior performance of a human U6 promoter over a schistosome U6 promoter in cassettes driving shRNAs to silence reporter genes in schistosomes, at least in blood stage schistosomules of *S. mansoni*. We are interested to establish long-term, stable and transgenerational RNAi to facilitate investigation of the essentiality of schistosome genes which could, in turn, be targeted in new approaches for treatment and control of schistosomiasis. The human U6 gene promoter clearly represents a potentially valuable transgene element for shRNA studies in schistosomes. High throughput, vector-based RNAi analysis can be expected to advance functional genomics for schistosomes in assisting the discovery of novel targetable genes (Stefanic et al. 2010).

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ANEXO II

En el siguiente apartado se incluyen trabajos de revisión publicados en revistas arbitradas internacionales en los cuales he participado como colaborador durante el transcurso de este trabajo.

- Cultivo para la manipulación genética de diversos estadios de desarrollo de *Schistosoma mansoni*

  Victoria H. Mann, María E. Morales, **Gabriel Rinaldi**, Paul J. Brindley
  Parasitology (2010), 137, 451–462.

- Symposium : Generando schistosomas transgénicos

  Victoria H. Mann *, Sutas Suttiprapa*, **Gabriel Rinaldi** *, Paul J. Brindley1
  (* Igual contribución al trabajo)
  PLoS Neglected Tropical Diseases (2011) 5(8): e1230

- Manipulación génica de schistosomas- progreso con vectores integrables

  Sutas Suttiprapa, **Gabriel Rinaldi**, Paul J. Brindley
  Parasitology (2011), in press. doi:10.1017/S003118201100134X

Culture for genetic manipulation of developmental stages of *Schistosoma mansoni*

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SUMMARY

Genomes of the major human helminth parasites, and indeed many others of agricultural significance, are now the research focus of intensive genome sequencing and annotation. A draft genome sequence of the filarial parasite *Brugia malayi* was reported in 2007 and draft genomes of two of the human schistosomes, *Schistosoma japonicum* and *S. mansoni* reported in 2009. These genome data provide the basis for a comprehensive understanding of the molecular mechanisms involved in schistosome nutrition and metabolism, host-dependent development and maturation, immune evasion and invertebrate evolution. In addition, new potential vaccine candidates and drug targets will likely be predicted. However, testing these predictions is often not straightforward with schistosomes because of the difficulty and expense in maintenance of the developmental cycle. To facilitate this goal, several developmental stages can be maintained *in vitro* for shorter or longer intervals of time, and these are amenable to manipulation. Our research interests focus on experimental studies of schistosome gene functions, and more recently have focused on development of transgenesis and RNA interference with the longer term aim of heritable gene manipulation. Here we review methods to isolate and culture developmental stages of *Schistosoma mansoni*, including eggs, sporocysts, schistosomules and adults, in particular as these procedures relate to approaches for gene manipulation. We also discuss recent advances in genetic manipulation of schistosomes including the deployment of square wave electroporation to introduce reporter genes into cultured schistosomes.

Key words: Schistosome, sporocyst, schistosomule, egg, *in vitro* culture, electroporation.

SCHISTOSOME GENOMICS

The helminth parasites of humans belong to the phyla Platyhelminthes (the flatworms) and Nematoda (the roundworms). The flatworms include flukes (trematodes) and tapeworms (cestodes). Schistosomes are blood flukes – the adult forms reside in the venules of the intestines (*Schistosoma japonicum*, *S. mansoni*) or urogenital system (*S. haematobium*). Unlike other platyhelminths, schistosomes are dioecious, and exhibit marked sexual dimorphism as adults. Advances in molecular genetics and immunology hold the promise to control the spread of schistosomiasis and to combat the morbidity and mortality associated with this great neglected tropical disease (Hotez *et al*. 2008). Control of schistosomiasis largely relies on chemotherapy with praziquantel, and the wide-scale use of this drug has led to concerns that drug resistance might develop (see Utzinger and Keiser, 2004). As a consequence, the WHO *Schistosoma* Genome Project was initiated in the early 1990s with the declared goal to identify target genes in order to be able to address fundamental questions on the biology and physiology of the parasites, and to guide the rational development of new interventions (Hu *et al*. 2004; Han *et al*. 2009). Schistosomes have comparatively large genomes, estimated at 398 megabase pairs (MB) for the haploid genome of *S. japonicum* (*Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium *et al*. 2009) and 363 MB for *S. mansoni* (Berriman *et al*. 2009) arrayed on seven pairs of autosomes and one pair of sex chromosomes. *S. haematobium*, the other major schistosome species parasitizing humans probably has a genome of similar size, based on similarity of the karyotypes (Hirai *et al*. 2000). The schistosome genomes represent the first of any of the Lophotrochozoa to be published. Analysis of the genomes revealed widespread domain structure reduction, complex signal transduction and sensory pathways, proliferation of mini-exons, unusual intron size distribution, large

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numbers of protease encoding genes, and other singular features (*Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium *et al*. 2009; Berriman *et al*. 2009). The reports of the draft genomes of *S. japonicum* and *S. mansoni* followed the reasonably comprehensive descriptions of the transcriptomes and proteomes (Hu *et al*. 2003; Verjovski-Almeida *et al*. 2003; Liu *et al*. 2006, 2008; Jolly *et al*. 2007; Hokke *et al*. 2007; Gobert *et al*. 2009). Less is known about *S. haematobium* although since more people are infected with this pathogen than both *S. japonicum* and *S. mansoni* combined (see Hotez *et al*. 2008), and because it also causes bladder cancer (Bouvard *et al*. 2009), genomics and transcriptomics study of *S. haematobium* clearly deserves more attention.

CULTURE METHODS, MOLECULAR TOOLS TO INVESTIGATE GENE FUNCTION

Despite this abundance of sequence data, functional analysis of potential target genes will not be possible until reliable methods for reverse genetics in schistosomes become available. Whereas reverse genetics tools including gene silencing by RNAi and transgenesis are finding utility in schistosomes (Morales *et al*. 2008; Rinaldi *et al*. 2009) *in vitro* culture techniques remain necessary to undertake these gene manipulations and other approaches. Transformation and gene manipulation in schistosomes have been reviewed (e.g. Beckmann *et al*. 2007; Brindley and Pearce, 2007) and informative reviews are available also on the general maintenance of the schistosome life cycle (e.g. Hackett, 1993; Lewis, 1998). To date, tools and protocols for gene manipulation of schistosomes remain rudimentary, especially when compared to established transgenesis systems and techniques in other pathogens such as some parasitic protozoa and for vector mosquitoes (Crabb and Gilson, 2007; Terenius *et al*. 2008), as well as in mammalian systems (e.g. Sasaki *et al*. 2009). The fundamental nature of the blood flukes – the complex developmental cycle, large size, multicellular tissues and complex organization, and the absence of immortalized cell lines and inability to rear the entire life cycle *in vitro*, have hindered development of tractable transgenesis models. Although the entire developmental cycle of the human schistosomes cannot be maintained *in vitro*, laboratory maintenance of the developmental cycles of all three human schistosomes can be accomplished using rodents as the mammalian hosts and natural host snail species as the intermediate hosts (Lewis, 1998). In addition, a number of the developmental stages, both mammalian and molluscan parasitic stages can be maintained in the laboratory.

Schistosomes are large, multicellular eukaryotes, and though aceolomate, they possess complex organ systems including a gut, female and male reproductive tissues, muscles, nervous tissues with eyespots and so forth. The blood-stage forms are covered by a syncytial tegument that is bounded at the parasite-host interface with a double lipid bilayer. Furthermore, the developmental stages differ dramatically in appearance and structure, cell numbers, ratio of germ to soma, and morphology. All these features pose challenges for genetic manipulation and certainly for germ line transgenesis. However, genetic manipulation and germ line transgenesis are worthwhile goals because they would facilitate a deep understanding of the molecular biology of schistosomes, roles of molecules in host-parasite interaction and, ultimately, to identify molecules that could be targeted/disrupted with drugs or vaccines. Here we review recent approaches to maintenance of developmental stages of *S. mansoni*, specifically in relation to genetic manipulation of these parasites for subsequent investigation of genetic transformation and transgenesis (Kines *et al*. 2006, 2008; Morales *et al*. 2007) as well as transient gene silencing by RNAi (Morales *et al*. 2008; Rinaldi *et al*. 2009).

ADULTS

S. mansoni worms commence shedding eggs from 42 days after infection of mice; many eggs are discharged with the faeces although a large percentage of all eggs produced eventually become entrapped in the capillary beds of the liver and other organs. We recover adult *S. mansoni* from experimentally infected mice six to 12 weeks after infection by portal perfusion, as described (e.g. Smithers and Terry, 1965; Duvall and DeWitt, 1967; Lewis, 1998). In brief, we euthanize infected mice with an overdose of sodium pentobarbital administered along with heparin into the peritoneal cavity (Duvall and DeWitt, 1967). This causes the schistosomes to shift from the mesenteric veins to the liver and inhibits blood clots, both of which enhance efficiency of perfusion of the worms from the vasculature. After death, the mouse is thoroughly wetted with 70% ethanol using a pump-spray to minimize potential for microbial contamination (and contamination with mouse hair) in the subsequent procedures. We make an incision with scissors in the abdomen after which the skin is peeled back to expose the peritoneum. Thereafter we cut through the rib-cage and diaphragm to expose the heart, and position the mouse over a 9 cm diameter Petri dish in order to collect adults soon to be flushed from the mesenteric veins. The hepatic portal vein (enlarged in schistosome-infected mice because of portal hypertension) of the mouse is located and severed with scissors. Occasionally, a bolus of adults immediately comes out of the vein and it is convenient to rinse the mouse viscera close to the portal vein in order to collect them in order to recover as many adult worms as possible before the

Table 1. Culture conditions and media for developmental stages of *Schistosoma mansoni*

Developmental stage	Medium	Atmosphere/Temperature	Key references
Egg	Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS), 1× penicillin and streptomycin	5% CO_2 in air; 37 °C	Rinaldi *et al*. (2009)
Primary sporocyst	MEMSJ-E	5% CO_2, 5% O_2; 90% N_2; 27 °C	Kawanaka *et al*. (1983, 1985)
Schistosomule	Modified Basch's medium supplemented with human erythrocytes	5% CO_2 in air; 37 °C	Basch (1981) Morales *et al*. (2008)
Adult	Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 1× antibiotic/antimycotic, and human erythrocytes	5% CO_2 in air; 37 °C	Kines *et al*. (2008)

perfusion. We insert a 20-gauge needle fitted to (6·35 mm internal diameter) tubing from a peristaltic pump (e.g. Simon Varistaltic pump model no. 72-312-000), connected to a reservoir of at least one litre of perfusion buffer (150 mM sodium chloride, 15 mM sodium citrate, pH to 7·0 with sodium hydroxide) (= 1 × SSC) into the left ventricle. We inject perfusion buffer into the heart at a flow rate of ∼5 ml per min and perfuse the vasculature of the mouse with ∼10 ml buffer. If the portal perfusion is effective, the liver changes from a deep maroon to greyish colour. The adult schistosomes are discharged from the severed hepatic portal vein into the Petri dish along with the blood and perfusate. A second perfusion can be undertaken in order to recover as many adult worms as possible. After perfusion, we rinse the mouse viscera with additional perfusion buffer (∼2 ml discharged from the pump) with the aim of flushing out additional adult schistosomes that may have been perfused from the portal system but which lodged on the exterior of the viscera or within the peritoneal cavity. We remove the liver from the perfused mouse, taking care not to perforate the stomach or intestines, and transfer the liver to a sterile 50 ml conical tube.

After the infected mice have been necropsied and the schistosomes perfused, we transfer the pooled perfusion buffer, adult schistosomes and blood to a sterile beaker (e.g. 600 ml beaker) until the adults can be prepared for *in vitro* culture. The adults should be removed as soon as practical (preferably within an hour) from the bloody perfusion buffer. We wash the worms by gravity using phosphate buffered saline (1× PBS) with 1× antibiotic/antimycotic (Invitrogen, Carlsbad, CA, catalogue no. 15240-062). This is performed several times until the PBS is apparently free of blood and other visible contaminants such as mouse hair. Thereafter, we culture the worms in DMEM supplemented with 10% foetal calf serum (FCS) serum and 1× antibiotic/antimycotic (Invitrogen) under 5% CO_2 in air at 37 °C (Table 1). For long-term culture (> two days), we supplement the culture of adult schistosomes with washed human erythrocytes, e.g. one μl erythrocytes (50% suspension)/ml culture medium (Kines *et al*. 2008). In general, we culture from several to 20 adult worms (mixed sexes) in 3–5 ml of culture medium in 6-well plates (Sarstedt Inc, Newton NC, catalogue no. 83.1839).

EGGS

This method is based on the procedure of Dalton *et al*. (1997). We carry out the procedures in a biological safety cabinet in order to enhance aseptic cultivation of the eggs. We transfer livers from infected mice at necropsy to a 50 ml conical tube (above), including three to five infected livers per tube. We fill the tube to the 40 ml mark with perfusion buffer and retain the tube on ice until all the livers are processed. Afterwards, we remove the livers from the conical tube with sterile forceps, and if possible, remove the gall bladder and fibrous tissues. We transfer the liver to a 9 cm Petri dish that contains ∼5 ml 70% ethanol where the liver is rinsed thoroughly. We replace the dish and ethanol for each liver. After this, we rinse the liver in Dulbecco's phosphate buffered saline (DPBS) supplemented to 1× antibiotic/antimycotic, and then transfer the livers to a sterile Petri dish. Here we chop/mince the liver to a fine consistency using a sterile razor blade or scalpel (we often process 10 livers at a time), and then transfer the chopped livers to a 50 ml conical tube, three to five chopped livers per tube. We also rinse the Petri dish with DPBS and transfer this wash to the chopped livers in the 50 ml tube. We fill the tube to the 40 ml mark with DPBS, then add 5 ml of 5% clostridial collagenase solution (25 mg) (Sigma-Aldrich, St. Louis, MO, catalogue no. C5138). The collagenase is included to release the

eggs from granulomas by proteolysis of the interstitial matrix of mouse liver tissue. We prepare and sterile filter the collagenase freshly each time. We also include 500 μl of polymixin B (100K Units) (Sigma-Aldrich, P4932-1MU), a gram negative bactericidal antibiotic. If processing fewer livers, we adjust the concentrations of collagenase and polymixin B accordingly. We cap the 50 ml tube tightly and seal the exterior of cap with several layers of tightly bound Parafilm. The capped tube containing the liver/collagenase digest is incubated at 37 °C for 15–18 hours, with gentle shaking. Subsequently, we centrifuge the tube containing the digested livers at 400 × *g* for 5 min at RT. After decanting the supernatant, we resuspend the pelleted material (containing the schistosome eggs) by refilling the tube to 50 ml with DPBS containing 1× antibiotic/antimycotic. (We have seen liver cells on top of the pellet of eggs; these can gently decanted without appreciable loss of schistosome eggs.) We resuspend the pellet material containing the eggs by refilling the tube to 50 ml with DPBS containing 1× antibiotic/antimycotic, and repeat the wash procedure three more times. Afterwards, the pellet is resuspended in 25 ml DPBS. Using a 10 ml serological pipette, we force the suspension through a sterile 250 μm sieve (Arthur H. Thomas Co., Philadelphia, PA) into a sterile beaker (e.g. 600 ml beaker). The resulting filtrate is forced through a 150 μM sieve (Arthur H. Thomas Co.) into a second sterile beaker, transferred to a 50 ml conical tube, and centrifuged at 400 × *g* for 5 minutes at room temperature. In parallel, we prepare a Percoll column: 8 ml of sterile Percoll mixed with 30 ml of sterile filtered 0·25 M sucrose in a 50 ml conical tube. After the filtrate is centrifuged, we decant the supernatant and add three ml of DPBS to the pellet. The resultant slurry is mixed thoroughly after which it is applied gently, using a serological pipette, to the top of the Percoll gradient, minimizing disruption to the surface of the Percoll/sucrose solution. The Percoll gradient is centrifuged at 800 × *g* for 10 minutes in a swinging bucket rotor (e.g. Eppendorf 5810 R, rotor A-4-62). By aspiration starting at the top, we remove the surface layer of the gradient that contains liver cells. We transfer the eggs (pelleted at the bottom of the tube) to a new tube and resuspend in DPBS. These are washed three times in DPBS, and then resuspended in 0·5 ml DPBS. We apply this to the surface of a second Percoll column, prepared by combining 2·5 ml Percoll with 7·5 ml of sterile filtered 0·25 M sucrose in a 15 ml conical tube. The eggs are centrifuged through this second Percoll column, and washed as above. We remove as much of the DPBS as practical, and suspend the eggs in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS) and 1× penicillin/streptomycin (Invitrogen, catalogue no. 15140-122). We split the eggs into 2 ml aliquots in a 6-well plate and culture at 37 °C, 5% CO_2 (Fig. 1A–D) (Table 1).

Dalton *et al*. (1997) demonstrated using Southern hybridization analysis that eggs purified with this Percoll-based procedure are free of detectable contaminating host tissues, and therefore can be confidently employed in molecular studies. The eggs can be maintained in culture for a maximum of 10 days in DMEM media containing 10% FBS and 1× penicillin/streptomycin. We recommend carrying out manipulations of cultured eggs before they reach seven days age of culture. After this, they begin to lose viability; after three weeks in culture, we have observed they will not hatch upon transfer to distilled water (below).

MIRACIDIA

To isolate miracidia, we resuspend the *S. mansoni* eggs in 5 ml sterile distilled H_20 (dH_20) at 23 °C, 1000 to 2000 eggs per ml. The eggs can be aliquoted in one ml volumes into the wells of a sterile 24-well tissue culture plate (Sarstedt), after which an additional one ml of dH_20 can be added to the well. We then locate the 24-well plate directly under a fluorescent light (low heat) source to induce hatching of the eggs (Lewis, 1998) (Fig. 2A, B). We remove one ml of supernatant from the surface (to avoid non-hatched eggs) every 30 min or so, using a pipette, and transfer water containing miracidia into a 50 ml conical tube, on ice. This process is continued at 30 minute intervals for two to three hours to harvest miracidia. After the final harvest, the miracidia can be counted; they will have settled to the bottom of the 50 ml tube because of the ice cold temperature. However, if they remain non-concentrated, the tube can be centrifuged at 800 × *g* for 10 min at 4 °C after which the supernatant is removed quickly by suction pipetting. At that point, we resuspend the miracidia in a minimal volume of dH_2O, where they can be counted by sampling several small aliquots, e.g. 5 μl miracidia added to 15 μl 95% ethanol, counted using a microscope.

SPOROCYSTS

To establish cultures on *in vitro* transformed sporocysts, we transfer miracidia from water (above) into sporocyst media in 6-well plates (Sarstedt) at a concentration of 500 to 2000 miracidia per well. For this, we employ a chemically defined medium termed MEMSE-J (Kawanaka *et al*. 1983, 1985) (Table 1). The formula for this medium is presented in Table 2; we prepare MEMSE-J in one litre batches, and store in aliquots of 50 ml at −20 °C until needed. Regardless of the concentration of the miracidia, we include $\leqslant$500 μl of water containing the miracidia into the 6-well plate, and increase the volume per well to 5 ml with sporocyst medium. We maintain the 6-well plate containing the developing sporocysts in a hypoxia chamber (e.g. Billups-Rothenberg,

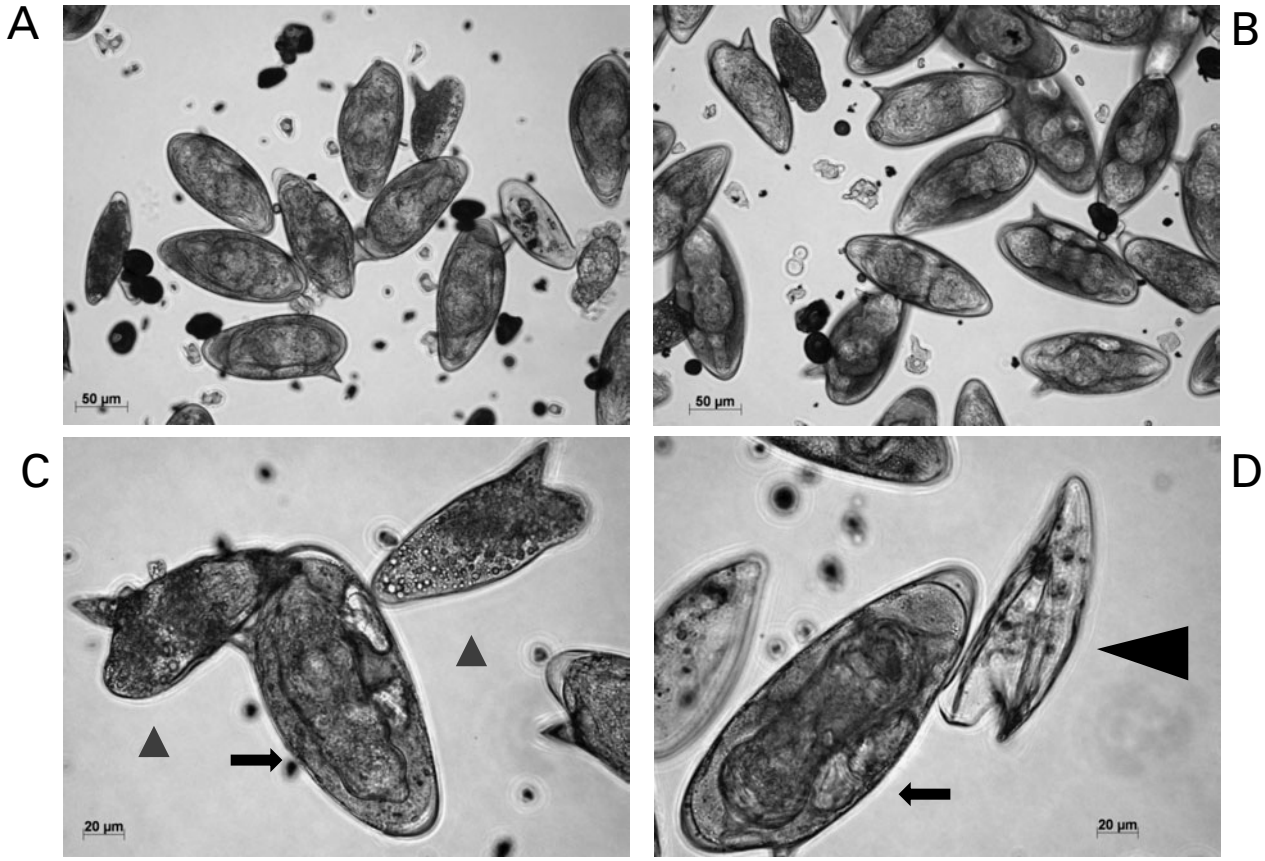

Fig. 1. Cultured eggs of *Schistosoma mansoni*. These eggs were isolated from livers of experimentally infected mice. Panel A and B: micrographs illustrating a mixed population, mature and immature eggs, three days after isolation from mouse liver. Scales bars = 50 μm. Panel C: two immature eggs (black arrow-heads) and a mature egg containing the miracidium (black arrow); scale bar = 20 μm. Panel D: a mature egg containing miracidium (black arrow) and an empty egg shell after hatching has taken place (black arrow-head); Scale bar = 20 μm. The micrographs were taken using a digital camera (Zeiss AxioCam ICc3) fitted to an inverted microscope (Zeiss Axio Observer A1).

Del Mar, CA catalogue no. MIC-101) under 5%O_2, 5% CO_2, 90%N_2 at 27 °C (Bixler *et al*. 2001). It is important to maintain the atmospheric pressure in the chamber below 2 psi (<15 kPa) in order not to damage the chamber's atmospheric seal. After the miracidia have shed the ciliated plates (Fig. 2B–D), at which point they are referred to as *in vitro* cultured sporocysts, they can be maintained for at least 14 days. In addition, other investigators have maintained *in vitro* cultured sporocysts in co-cultures with Bge cells (ATCC, Manassas, VA, catalogue no. CRL-1494), a cell line from the pulmonate snail *Biomphalaria glabrata* (see Coustau and Yoshino, 2000), or Bge conditioned culture medium. This can facilitate longer term, perhaps continuous, culture of the sporocysts and even production of cercariae (Ivanchenko *et al*. 1999), although this is challenging. *In vitro* cultured sporocysts can be transplanted into *B. glabrata* snails to establish lines of *S. mansoni* (see Kapp *et al*. 2003).

SCHISTOSOMULA

We obtain *Biomphalaria glabrata* snails infected with the NMRI (Puerto Rican) strain of *Schistosoma mansoni* from Dr. Fred Lewis, Biomedical Research Institute (Rockville, MD) and culture the snails in aquaria at 25 °C. Cercariae begin to exit infected snails into the aquarium water from about 35 days after infection with miracidia (Lewis, 1998). We wash the shells of the infected snails with deionized water (or aged tap water) delivered in a jet stream from a domestic garden-type weed sprayer to remove rotifers. For this task, we transfer the snails from aquaria into a kitchen-style mesh colander, and pressure spray them over a sink in order to remove rotifers and other commensals that tend to establish on the shells of the snails. Rotifers release metabolites injurious to schistosome cercariae (Stirewalt and Lewis, 1981). Subsequently, the snails are transferred to 100–200 ml of de-chlorinated water, and the snails in the beaker illuminated from about 20 cm above with a fluorescent light (60 watt equivalent) source. After one to two hours, the water containing the cercariae is transferred into a second sterile beaker avoiding any debris in the bottom of the beaker. To remove residual debris, we pour the cercarial water through a stainless steel screen (e.g. Millipore catalogue no. XX1004730, 47 μm) into a 50 ml conical tube(s). The snails are returned to aquaria.

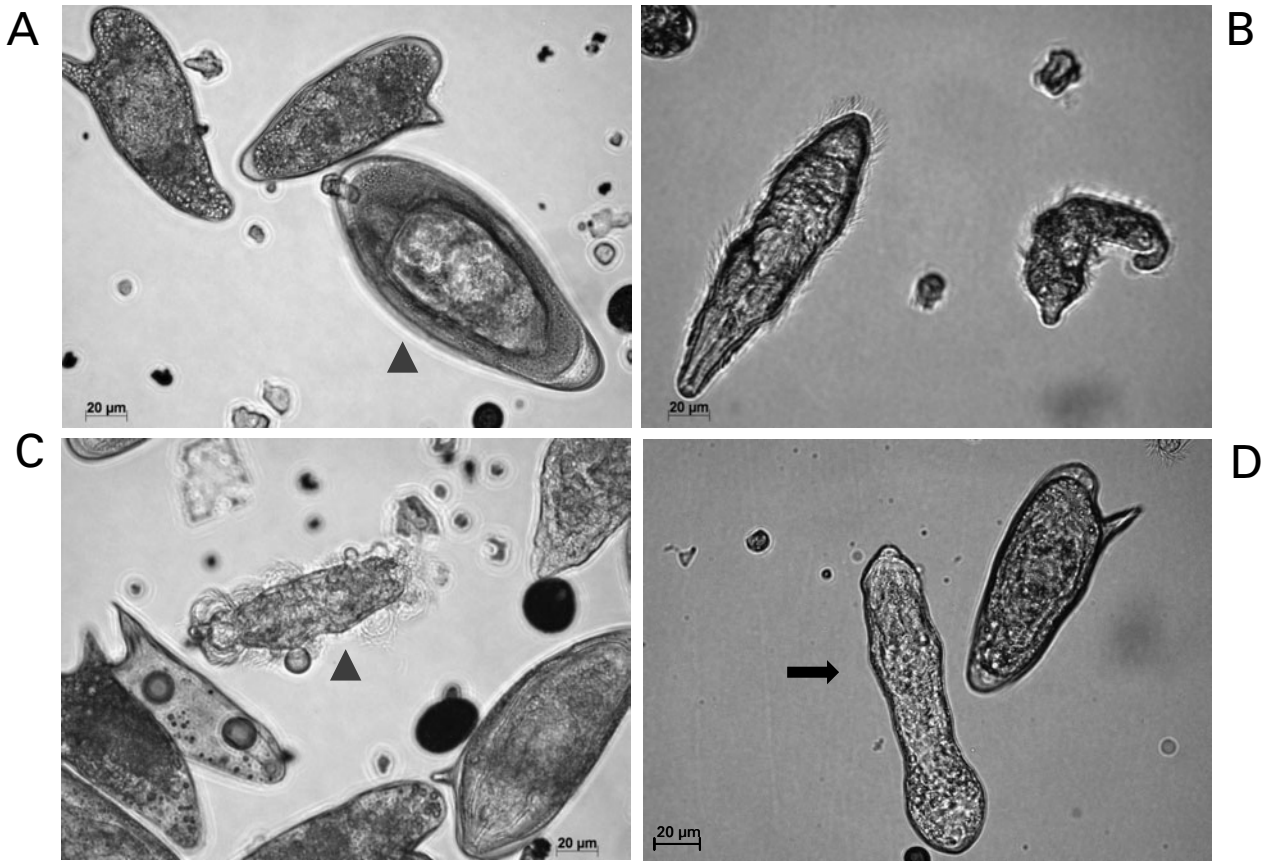

Fig. 2. Miracidia of *Schistosoma mansoni* hatching from eggs and transforming into mother sporocysts *in vitro*. Panel A: mature egg ready to hatch (arrow-head) and two immature eggs. Panel B: two miracidia after hatching. Panel C: miracidium losing its ciliated plates (arrow-head). Panel D: *in vitro* cultured mother sporocyst (black arrow). Scale bars: 20 μm. The micrographs were taken using a digital camera (Zeiss AxioCam ICc3) fitted to a Zeiss Axio Observer A1 microscope.

We concentrate the cercariae by centrifugation ($400 \times g$/10 min) and wash them $\times 3$ with somule wash, DPBS supplemented with $1\times$ antibiotic/antimycotic or similar wash buffer, e.g. Correnti and Pearce (2004). The cercariae are resuspended in $\sim$4 ml of somule wash in a sterile Petri dish. A 22-gauge double-hub, emulsifying needle with a stabilizing bar (Popper & Sons, New Hyde, New York, catalogue no. 7975) is fitted to a sterile 10 ml Luer-lock syringe. A second 10 ml syringe is used to draw up the cercariae. An additional three ml of somule wash is added to the Petri dish and this is also drawn up into the syringe. This syringe is fitted to the open end of the emulsifying needle. The tails of cercariae are sheared off by $\sim$20 passes back and forth through the emulsifying needle. (Because of obvious biohazard with this procedure, which involves manipulation of thousands of cercariae, we recommend that protective clothing and gloves, including protection for the face and the eyes be worn.) Thereafter, the schistosomule bodies are isolated from the sheared tails by Percoll gradient centrifugation (Lazdins *et al*. 1982). After the cercariae have been sheared, the contents of the syringes are dispensed into a 15 ml conical tube and concentrated by centrifugation $450\times$ *g* for 5 min. The Percoll gradient is prepared in 15 ml conical tubes; 10 ml of the Percoll gradient solution is added to each tube (12 ml Percoll, 2 ml $10\times$ PBS, 0·5 ml 100 mM Hepes, 0·5 ml antibiotic-antimycotic, 5 ml sterile water). The cercarial bodies [schistosomula] are resuspended in one ml somule wash. 0·5 ml of the schistosomula suspension is applied to each column carefully in order not, or only minimally, to disturb the surface of the Percoll gradient. We centrifuge the gradient at $450\times$ *g* for 10 minutes, with the brake at its least forceful setting (e.g. setting number 1). The tails will be at the top of the column, the schistosomule bodies at the bottom. Thereafter, we remove the top layers of the gradient first; the schistosomula should be pelleted at the bottom of the tube. (Cercarial tails can be retained and stored at $-80\,^{\circ}$C for later use, for example as a source of genomic DNA.) We wash the schistosomules three times in wash medium and transfer them to modified Basch's medium at 37 °C under 5% CO_2 in air (Tables 1, 3) (Basch, 1981). For procedures where we have investigated the activity of transgenes and/or schistosome gene promoters driving transgenes, we and others have cultured schistosomules for >15 days (Correnti *et al*. 2005, 2007; Morales *et al*. 2007) (Fig. 3A–D).

Table 2. Composition of MEMSJ medium[a] for culture of *Schistosoma mansoni* primary sporocysts

	Component[b]	Concentration (mg/L)
1.	L-Arginine	126
2.	L-Cysteine	24
3.	L-Histidine.HCL.H_2O	42
4.	L-Isoleucine	52
5.	L-Leucine	52
6.	L-Lysine.HCl	72·5
7.	L-Methionine	15
8.	L-Phenylalanine	32
9.	L-Threonine	48
10.	L-Tryptophan	10
11.	L-Tyrosine	36
12.	L-Valine	46
13.	L-Glutamine	300
14.	Glycine	10
15	L-Serine	46
16.	Choline chloride	3
17.	Nicotinamide	1
18.	NaCl	6800
19.	KCl	400
20.	$CaCl_2$	200
21.	$MgCl_2.6H_2O$	200
22.	$NaH_2PO_4.2H_2O$	150
23.	$NaHCO_3$	2000
24.	Phenol red	10
25.	Penicillin/streptomycin	1×
	NaOH to pH 7·2	

[a] Adapted from Kawanaka *et al*. (1983).
[b] Components 1–12 are the same as Invitrogen's 1× MEM amino acid solution (catalogue no. 11130-051).

Table 3. Composition of modified Basch's medium

Basch's medium (Basch, 1981)	Working concentration
Basal Medium Eagle (Invitrogen catalogue no. 21010)	to final volume
Lactalbumin hydrolysate	1 g/L
Glucose	1 g/L
Hypoxanthine	0·5 μM
Serotonin	1 μM
Insulin	8 μg/ml
Hydrocortisone	1 μM
Triiodothyronine	0·2 μM
MEM vitamins (Invitrogen cat. 11120-052)	0·5×
Schneider's medium	5%
Foetal bovine serum	10%
Hepes	10 mM
Antibiotic/antimycotic (Invitrogen cat. 15240-062)	1×

We change media on cultured schistosomules every second day, if feasible. For this, schistosomules are transferred to 15 ml conical tubes. The well is washed with somule wash and added to the tube until the tube contains ~10 ml. The tube is centrifuged for 5 min at 100 × *g* at RT or 4 °C. The supernatant is removed and the somules are resuspended in 4 ml of prewarmed somule media and returned to the 6-well plate. At day two or three after transformation from cercariae, washed human erythrocytes are included in the culture; 1–2 μl of red cells (50% suspension in DMEM) added to each well (Fig. 3). Once a week the old erythrocytes are removed and replaced with fresh blood cells. The somules are transferred from the plate to a 15 ml conical tube as above. The somules will settle by gravity for 30 minutes at 37 °C, after which the supernatant can be removed, the somules resuspended in ~4 ml warmed, fresh Basch's medium, and then returned to a new 6-well plate.

GENETIC MANIPULATION OF CULTURED SCHISTOSOMES

General considerations

The recent release of draft genome sequences of two of the major human schistosomes has underscored the pressing need to develop functional genomics approaches for these significant pathogens (*Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium *et al*. 2009; Berriman *et al*. 2009). Progress with functional genomics approaches and tools for schistosomes have been reviewed (Beckmann *et al*. 2007; Brindley and Pearce, 2007; Ndegwa *et al*. 2007; Mann *et al*. 2008). In particular, RNA interference (RNAi) has been developed to target numerous genes in *S. mansoni* and *S. japonicum* (e.g. Freitas *et al*. 2007; Morales *et al*. 2008; Ndegwa *et al*. 2007; Rinaldi *et al*. 2009). RNAi using short interfering RNAs (20 to 30 nucleotides) as well as longer double stranded RNAs (100 to more than 1000 nt) has been successful. In addition, the schistosome genome sequences make feasible genome-scale investigation of transgene integration into schistosome chromosomes. We have adapted several gene therapy approaches to transform schistosomes with the long-term aim of establishment of lines of transgenic schistosomes (Mann *et al*. 2008). These approaches include deployment of the *piggyBac* transposon and pseudotyped murine leukaemia retrovirus (Kines *et al*. 2006, 2008; Morales *et al*. 2007). Retrovirus- and *piggyBac*-mediated transductions both offer a means to establish transgenic lines of schistosomes, to elucidate schistosome gene function and expression, and to advance functional genomics approaches for the parasites. The theory, history and methods of binary transposons systems and retroviral/lentiviral approaches for transgenesis have been reviewed, e.g. Plasterk *et al*. 1999; O'Brochta *et al*. 2003; Miskey *et al*. 2005; Alfa and Blesch, 2006; Pfeifer and Hofmann, 2009. Given our recent experience with both RNAi and transgenesis approaches with *S. mansoni*, we have included here brief methods that facilitate transfer of foreign genetic materials and reporter genes into cultured schistosomes, including the deployment of square wave electroporation.

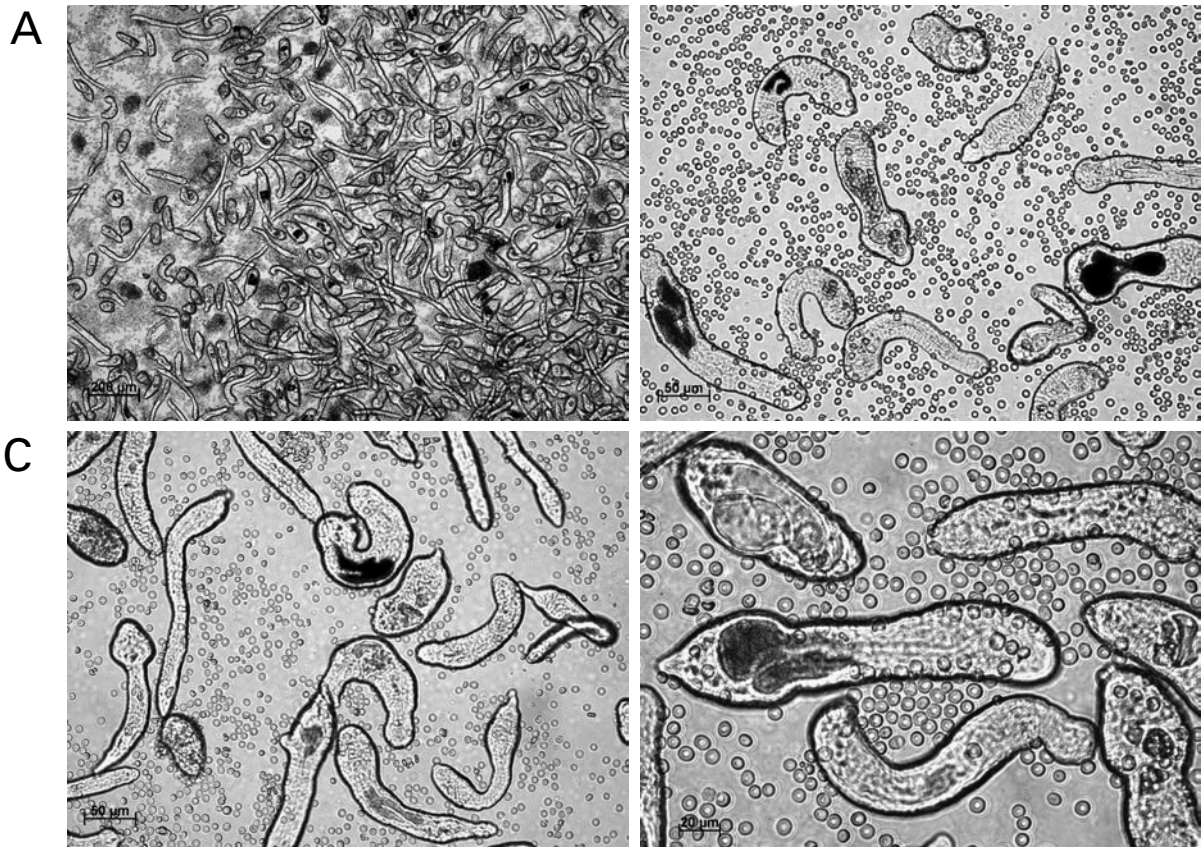

Fig. 3. Schistosomules of *Schistosoma mansoni*. The schistosomules were mechanically transformed from cercariae and cultured for 14 days. Black pigmented guts are evident in many of the larvae. Erythrocytes have been added to the cultures. Panels A to D: schistosomules viewed at 5, 20, 20 and 40× magnification, respectively. Scale bars are shown in each panel. The micrographs were taken using a digital camera (Zeiss AxioCam ICc3) fitted to a Zeiss Axio Observer A1 inverted microscope.

RNA interference

RNAi for schistosomes was pioneered by Skelly *et al*. (2003) and subsequently employed by many workers (see Ndegwa *et al*. 2007). Developmental stages of schistosomes including sporocysts and adults have been probed with dsRNA or short interfering RNAs (siRNAs) in RNAi investigations (e.g. Delcroix *et al*. 2006). Electroporation of dsRNA/siRNA, rather than soaking alone, appears to be much more efficient for schistosomules and adult *S. mansoni* worms (Ndegwa *et al*. 2007). Recently, we developed methods to assess for the presence of a viable RNAi pathway by silencing the exogenous reporter gene, firefly luciferase. We established the method in *S. mansoni* and thereafter confirmed its utility in the liver fluke *Fasciola hepatica*. This straightforward reporter system could provide investigators with a means to test the presence of a functional RNAi pathway in other parasites that are by other means intractable (Rinaldi *et al*. 2008).

Double stranded RNA (dsRNA) has been introduced into schistosome eggs for RNAi analysis (Freitas *et al*. 2007; Rinaldi *et al*. 2009). After the isolation of eggs from mouse livers (above), we have cultured eggs in a small volume of DMEM complete media as described above, at 37 °C under 5% CO_2 in 24 well-plates (~5000 eggs per well). For RNAi studies, in our case with investigation of proteolytic enzymes of eggs, we have soaked eggs in 20 μg/ml of dsRNA. Provided that enough dsRNA is available, we change the culture media supplemented with the dsRNA daily or every second day, with frequent microscopic examination to identify visual phenotypic effects (if relevant). We harvested eggs after seven days in culture with dsRNA, for subsequent RNA and biochemical activity analyses (Rinaldi *et al*. 2009). After one week in culture, eggs retain the capacity to hatch when transferred to distilled water (Figs. 1, 2) (above).

Electroporation

For electroporation of dsRNA, the schistosomula can be removed at 3 h and at 7, 9 and 11 days, or at other suitable times, after cercarial transformation. We rinse cultured developmental stages of schistosomes free of erythrocytes and/or serum by three or more washes in somule wash medium. At the time of electroporation, dsRNA is added at a final concentration 0·1 μg/μl to 3-h-old schistosomules, and at a final concentration 0·3 μg/μl for 7-, 9- and

11-day-old somules. We have accomplished electroporation using a single 20 ms square wave pulse at 125 V in 4 mm gap cuvettes (BTX, San Diego, CA) using a BTX ElectroSquarePoratorTM ECM830 with ~2000 schistosomules suspended in 100 μl of wash medium containing 10 to 30 μg of dsRNA. Immediately following electroporation, we transfer the parasites to pre-warmed Basch's medium (Table 3) and maintain in culture, as above (Correnti and Pearce, 2004; Correnti *et al*. 2005; Morales *et al*. 2008). After electroporation, we also have used the transformed schistosomes to infect mice. For example, we have resuspended 500 to 2000 in ~200 μl Modified Eagle's Medium and injected them into the thigh muscle or the peritoneal cavity of mice using a 22-guage needle (James and Taylor, 1976; Morales *et al*. 2008).

Transposons

In addition to dsRNA/siRNA, we have introduced binary transposon constructs into schistosomes by square wave electroporation (Morales *et al*. 2007). For electroporation in transposon studies using donor plasmids and helper (transposase) as mRNA, the schistosomules were removed from culture 15 days after cercarial transformation if expression of the reporter gene is important. Otherwise the schistosomules could be electroporated at younger age (Correnti *et al*. 2005; Morales *et al*. 2008). In brief, transposase mRNA was added to the DNA at a final concentration of 6 μg mRNA/100 μl. Electroporations were accomplished in 4 mm gap cuvettes (BTX) with ~1000–2000 parasites (somules, sporocysts or eggs) or 15–20 adults resuspended in 100 μl of wash medium containing 10–12 μg donor plasmid and 3–6 μg of helper mRNA using the BTX ElectroSquarePorator^{TM} ECM830. We introduce either intact circular or linearized (donor) plasmids, with or without transposase mRNA, into schistosomules by square wave electroporation (125 V, 20 ms, 4 mm). Immediately after electroporation, parasites are transferred to pre-warmed Basch's medium and cultured as described above. Subsequently, schistosomula were washed three times with pre-warmed wash medium and treated with DNase (New England Biolabs, Ipswich, MA) for 1 h at 37 °C to remove any residual donor plasmids. Parasites were then washed three more times with wash media to remove DNase, employed to infect mice (see above), or stored at −80 °C.

Retroviruses

With retrovirus virions as the transgenesis vectors, we have observed that both soaking and electroporation techniques can effectively facilitate integration of transgenes into the schistosome genome. We have exposed eggs, sporocysts, schistosomules and adult stages to Moloney murine leukaemia retrovirus (MLV) virions pseudotyped with vesicular stomatitis virus glycoprotein (VSVG) (Clontech) or lentiviral virions pseudotyped with VSVG (Invitrogen) (Kines *et al*. 2006, 2008; P. Brindley and others, unpublished). Both these viruses are replication-incompetent and accordingly they can be employed using Biological Safety Level 2 (BSL2) containment. To introduce the virus into the schistosomes, we soak worms in the presence of pseudotyped virions or in addition subject the parasites to square wave electroporation. With soaking alone, we reduce the volume of media in a 6-well plate to one ml, and add one hundred μl of concentrated virus ($>1 \times 10^6$ transducing units) to the well. We disperse the virus by gentle rocking of the plate. We add polybrene (Sigma) to 6 μg/ml to facilitate virion attachment to the surface of the target parasites. (Polybrene neutralizes charge repulsion between retrovirus virions and sialic acid on the surface of cells [Davis *et al*. 2004]). After gentle mixing, we incubate the plate overnight at 37 °C under 5% CO_2 (for sporocysts, 27 °C, 90% N_2, 5%CO_2, 5%O_2) (Table 1). For electroporation of parasites, we remove the parasites from the 6-well plate, pellet them as above, and resuspend in 100 μl of Opti-Mem 1 (Invitrogen). We transfer them to a 4 mm gap cuvette; add 100 μl of virion preparation in Opti-Mem 1 medium and electroporate, as above. After electroporation, we transfer the schistosomes to a 6-well plate and add 800 μl of DMEM with 10% FBS and 100U of penicillin and streptomycin (Invitrogen) to the well. They are incubated overnight at 37 °C under 5% CO_2 unless the life stage is the sporocyst (see above). The following day, after soaking and/or electroporation, we pellet the parasites at 800 × *g* and wash 3 times in DPBS. Eggs tolerate this electroporation, can hatch afterwards, and the resulting miracidia will infect snails (V. Mann, unpublished). After exposure to retrovirus virions, we have returned developmental stages of schistosomes to culture in new media appropriate for the life stage (Table 1). After appropriate intervals of time in culture, virion-exposed schistosomes can be assessed for productive transduction using appropriate approaches such as anchored PCR (Morales *et al*. 2007).

CONCLUDING REMARKS

Because many standard tools and approaches are not yet available, and because the schistosome developmental cycle cannot be completed without animal hosts, *in vitro* culture methods and procedures have particular importance for genetic manipulation of schistosomes. Whereas the methods that are currently available are not optimal, they can facilitate advances in genetic manipulation which, in turn, will

enhance our understanding of schistosome physiology and the host-parasite relationship. Despite the limitations of maintenance of schistosomes *in vitro*, new insights into fundamental helminth biology are accumulating. Genetic manipulation approaches will gain momentum because of the newly available draft genomes and the application of RNAi and nascent transgenesis technologies. At the same time, much of our understanding of Th2-type immune responses, functions of regulatory T cells, generation of alternatively activated macrophages and the transmission dynamics of infectious agents, for example, are derived from schistosome and other parasitic helminth paradigms. Ultimately, advances in molecular and medical helminthology – including manipulation of the schistosome genome – can be expected to be translated into new interventions targeting schistosomiasis and other helminthiases, and/or therapeutics for other ailments (Hotez *et al*. 2008; Han *et al*. 2009; Hewitson *et al*. 2009).

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PLoS NEGLECTED TROPICAL DISEASES

Symposium

Establishing Transgenic Schistosomes

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Draft genome sequences for *Schistosoma japonicum* and *Schistosoma mansoni* are now available. The schistosome genome encodes approximately 13,000 protein encoding genes for which the function of only a small minority is understood. The new genes represent potential intervention targets. Molecular tools are needed to determine the importance of these new genes. There is a role for transgenesis in functional genomics of the new schistosome gene sequences, both in gain- and loss-of-function approaches such as insertional mutagenesis screens and vector-based RNA interference. This laboratory symposium focuses on the development of approaches, systems, and tools to address the problem of establishing transgenic schistosomes which, in turn, can be applied to fundamental questions of schistosome physiology, the host–parasite relationship, and to developing new interventions.

The Problem

Control of schistosomiasis largely relies on chemotherapy, but people rapidly become re-infected and the widespread use of praziquantel has led to concerns about development of drug resistance. Advances in molecular genetics, biochemistry, and vaccinology hold promise to control the spread of schistosomiasis and to combat the morbidity and mortality associated with this neglected tropical disease. Draft genome sequences for *Schistosoma japonicum* and *Schistosoma mansoni* are available [1,2]. Molecular tools are needed to determine the importance of newly identified genes. Problematically, few functional genomics tools are available for schistosomes. The potential value of transgenesis approaches for schistosomes is obvious given the progress made in model species and cell lines and indeed more tractable pathogenic species (e.g., [3–5]). There is a valuable role for transgenesis in functional genomics for investigation of schistosome genes. Devising tools to create transgenic schistosomes and deploying transgenic schistosomes in functional genomics analysis will advance knowledge of schistosomes and schistosomiasis.

Tutorial

Why Pursue Transgenesis for Schistosomes?

Transgenesis, including somatic and germ line approaches, is a desirable goal. It is a well-established approach for functional genomics in model species including *Caenorhabditis elegans* and *Drosophila melanogaster* (e.g., [6]). It should be able to facilitate gain-of-function and/or loss-of-function phenotypic and molecular analysis in schistosome parasites. Transgenesis approaches can facilitate vector-based RNA interference, and would be a potential forward genetic technology for insertional mutagenesis screens, which are feasible now that draft schistosome genome sequences are available. In addition, transgenes are potential tools for development of genetic therapy and/or vaccines. Approaches being developed for schistosome transgenesis include deployment of integration-competent vectors such as transposons and retrovirus. Integration-competent vectors are expected to lead to insertion of transgenes into schistosome chromosomes.

Which Vectors Can Be Used to Produce Transgenic Schistosomes?

Both integration-competent and non-integrating plasmids have been used to introduce transgenes into schistosomes [7–9]. Although both approaches have utility, there are compelling reasons to focus on integration-competent vectors, primarily because integrated transgenes can be propagated equally and reliably to the progeny of the transduced cell, including germ line cells. Integration-competent vectors include DNA transposons such as *mariner*, *Sleeping Beauty*, and *piggyBac*, and simple and complex retroviruses including murine leukemia viruses and lentiviruses such as HIV-1. Indeed, colleagues in our lab have demonstrated the proof of this principle by showing that the transposon *piggyBac* is transpositionally active in *S. mansoni* [8], and the vesicular stomatitis virus glycoprotein (VSVG)-pseudotyped murine leukemia retrovirus (MLV) can transduce *S. mansoni* and *S. japonicum*, leading to active proviral reporter transgenes integrated in the schistosome chromosomes [9–11]. Other approaches including deployment of bacteriophage integrases and fungal recombinases, which have found service in genome manipulation of, for example, *Plasmodium falciparum*, may also be of use [12,13], but have not yet been reported with schistosomes.

Which Developmental Stages Might Be Targeted?

Theoretically, the schistosome genome is targetable at any stage of parasite development given that for *S. mansoni*, for instance, the entire developmental cycle can be maintained in the laboratory in *Biomphalaria* species snails and the laboratory mouse (Figure 1). Some stages can be cultured ex vivo or in vitro, and returned to the snails or mice to continue development (see [14]). Other stages have potential advantages as targets for transgenes given their accessibility, tolerance to manipulation, size, and/or ratio of germ to soma (e.g., [10,15]). Also, schistosome stages are differentially accessible to delivery of transgenes, using ap-

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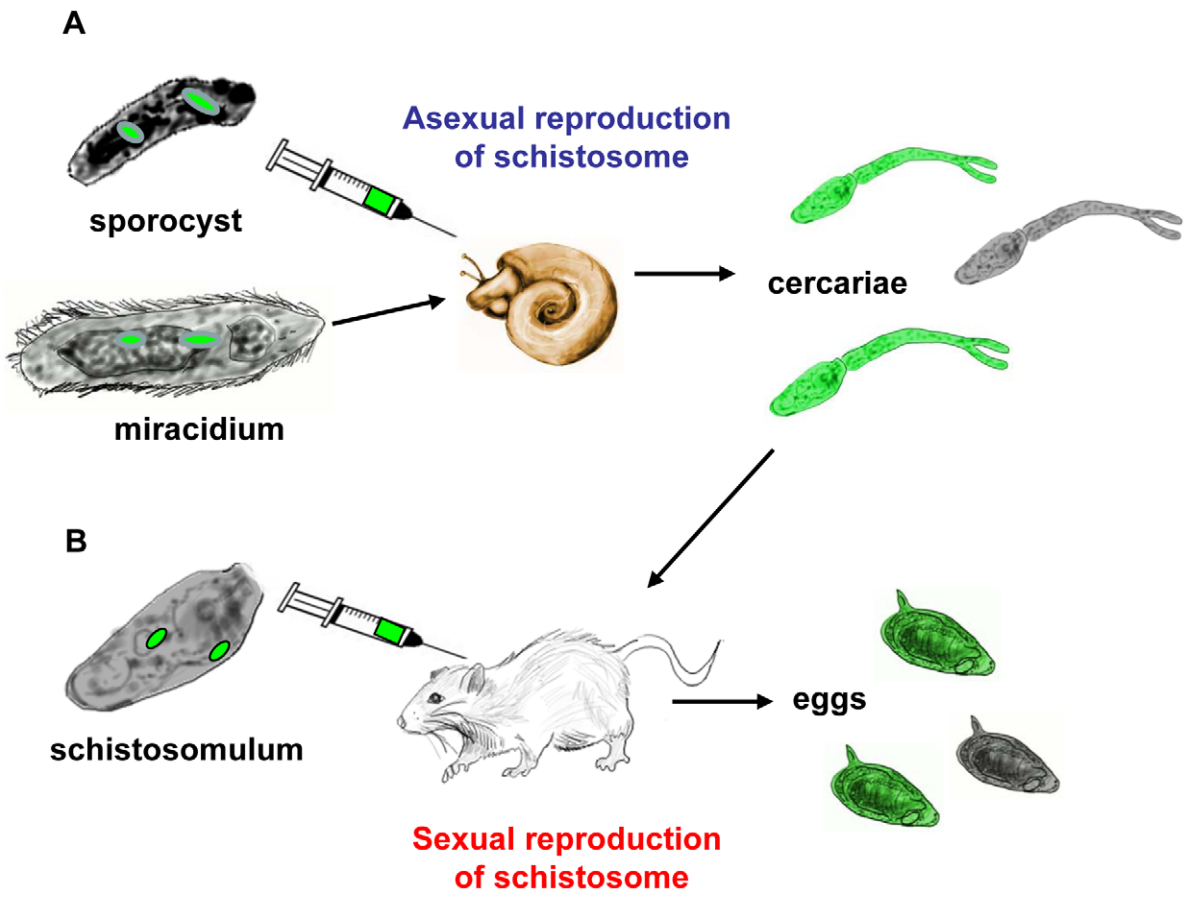

Figure 1. Schematic representation of general strategies that can be explored to introduce transgenes into the germ line involving the asexual (A) and sexual (B) reproduction processes of the developmental cycle of *S. mansoni*. In brief, eggs/miracidia, sporocysts, and/or schistosomula might be transduced by VSVG-pseudotyped Moloney murine leukemia retrovirus. Subsequently, snails can be infected with miracidia by the natural percutaneous route or with sporocysts by microinjection and mice infected by the parenteral route with transformed schistosomules or by the natural percutaneous route by cercariae. Progeny cercariae from snails and eggs from mice can be analyzed for transgenes and/or reporter transgene activities. The green colored cells in illustrations of larvae represent the potential presence of transgenes in germ line and/or somatic cells.
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proaches that have included particle bombardment, square wave electroporation, cationic polymer-based gene delivery, and infection of schistosomes with pseudotyped retrovirus [7,16,17]. Other approaches, including microinjection, should be of value, as indicated by progress with introduction of transgenes in parasitic nematodes [18]. The schistosome egg and the miracidium that hatches from the mature egg have desirable attributes for consideration in relation to transgenesis. These include the presence of the single cell zygote within the eggshell upon its release from the female blood fluke, high ratio of germ to somatic cells, ease of maintenance in vitro, accessibility of embryonic cells within the egg to transgenes of pseudotyped retrovirus virions, and ability of the miracidium, which is readily released from the mature egg by transfer of the cultured egg into sterile water to naturally infect the intermediate host snail [19,20]. The attributes also include availability of eggs from livers of experimentally infected rodents [7,14] and ability of the female to deposit viable eggs in vitro [21] (see below). Moreover, from a clinical perspective, the egg represents the major source of pathogenesis. Figure 1 outlines strategies that can explored to direct transgenes to the germ line of *S. mansoni* involving the asexual and sexual reproduction processes of the developmental cycle of the parasite.

Eggs and miracidia. In the first report of vertical or germ line transgenesis in schistosomes, Grevelding and colleagues [7] transfected miracidia with plasmid DNA encoding green fluorescent protein (GFP) driven by the schistosome actin gene promoter, and reported that the GFP transgene was transmitted to the F1 generation of miracidia, via passage through snails and mice. Transmission to the F2 and F3 generations was not apparent, though, likely because the transgene transmission as episomal and the extra-chromosomal transgene was diluted and/or lost in subsequent development and generations. Non-integrated, extra-chromosomal arrays of plasmid transgenes are the normal occurrence in transgenic *C. elegans* where transgene DNA assembles through non-homologous recombination into multi-copy concatemers or extra chromosomal arrays. These are inherited in non-Mendelian fashion. Nonetheless, integration into the *C. elegans* genome will improve transmission to the progeny (see [22]). Recently, we demonstrated the feasibility of manipulating eggs of *S. mansoni*. Eggs from mouse livers were soaked and/or electroporated with different reporter transgenes including MLV virions. Eggs were transduced with virions after which retroviral transgenes were detected and quantified in the genome of miracidia by real-time PCR [20].

In vitro laid eggs (IVLE). We have begun to focus on eggs laid in vitro by females aiming to target transgenes to the zygote or to the early blastula where the total number of cells would be less than in

the mature egg and where the germ to somatic cell ratio would be higher, which would enhance the likelihood of transfecting germ cell(s), a prerequisite for perpetuating an entirely transgenic schistosome or a mosaic form comprising transgenic and non-transgenic tissues. One approach to this goal involves culturing schistosomes recovered from mice and collection of eggs laid in vitro by these worms within 48 hours. (Eggs laid after the females have been in culture for >48 hours do not develop correctly [21].) With these in vitro laid eggs (we abbreviate as IVLE), we follow the protocol of Mann et al. [14], with modifications. We transfer adults into schistosomule medium and maintain them at 37°C immediately after perfusion from mice, and transfer the worms as soon as practicable into 74-μm diameter mesh netwell, 6-well plates (Fisher Bioscience, catalog no. 0720-0213). The worms are maintained in schistosomule medium in netwell plates for 48 hours after perfusion. IVLE and, occasionally, adult females fall through the mesh and collect on the bottom of the culture plate (Figure 2A). At 48 hours after perfusion of the worms from mice, we collect IVLE, and concentrate by filtering media containing IVLE through 0.8-μm mesh transwell (BD Biosciences, catalog no. 353097) (Figure 2B, 2C). Thereafter, IVLE are maintained in schistosomule medium where they develop and mature within 7 days (Figure 2D–2J). At that point, we transferred IVLE to sterile water, illuminated the culture with a bright lamp, and observed that many eggs (30% to 40%) hatch within 120 minutes. We observed that miracidia hatched from IVLE infected *Biomphalaria glabrata* snails from which, in turn, cercariae were released after 6 weeks (Figure 2K–2M). IVLE represents a tractable, developmental stage at which to target transgenes, especially since in developmental stages 0 and 1 (staging system of Jurberg et al. [19]), no cleavage of the zygote cell has yet taken place. In future studies, we plan to expose IVLE to pseudotyped MLV virions at the time eggs are released from the female schistosome, aiming to introduce transgenes into the schistosome germ line. (The studies with schistosome-infected laboratory mice were undertaken with the approval of the IACUC of The George Washington University, Washington, D.C.).

How Can We Increase the Likelihood of Chromosomal Integration?

For transposons, in particular for binary versions of broad host range vectors such as *piggyBac* and *Sleeping Beauty* (e.g., [4,8]), increased efficiency of integration can be accomplished using mRNA encoding the transposase rather than using helper plasmid, and further, optimal ration of transposon and transposase can be titrated. Also, transposase enzyme can be employed instead of mRNA of the gene

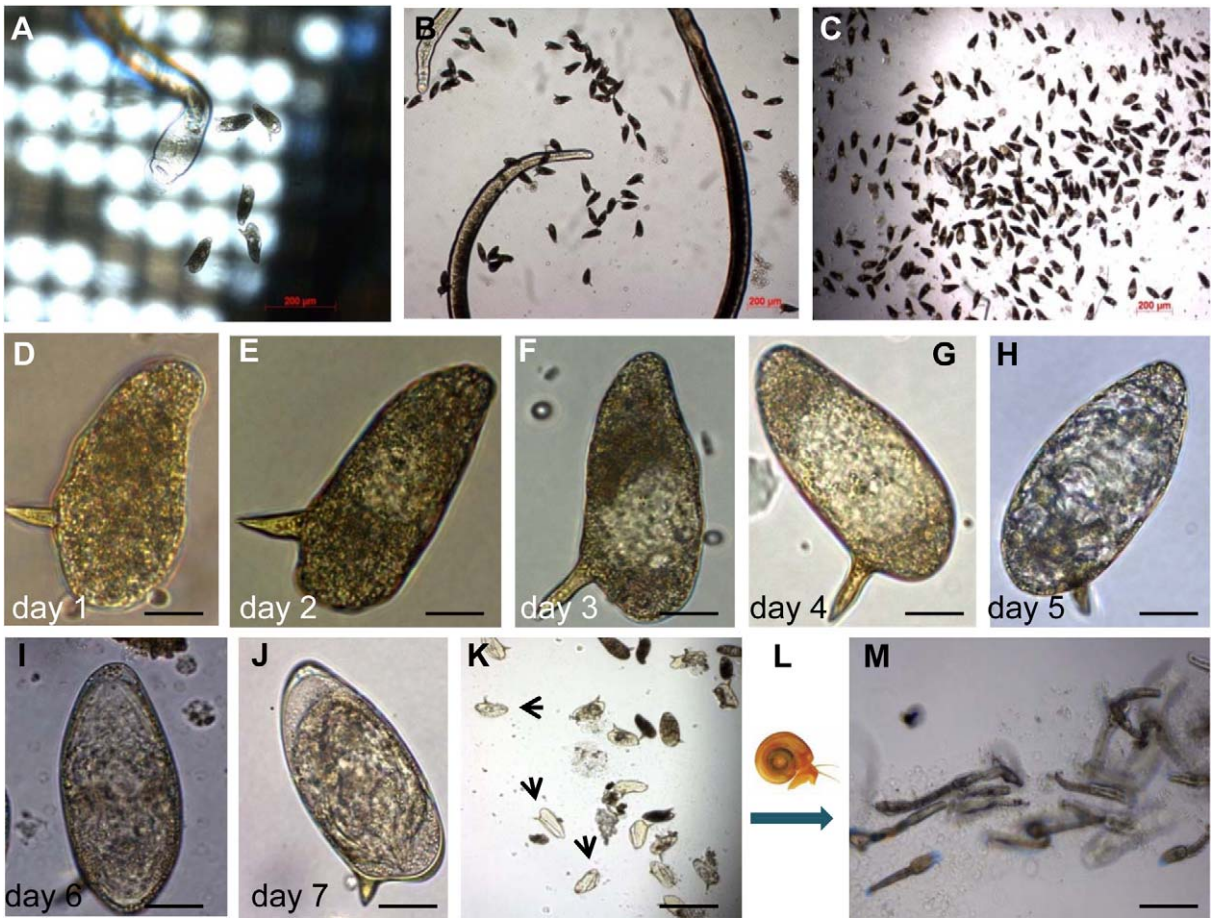
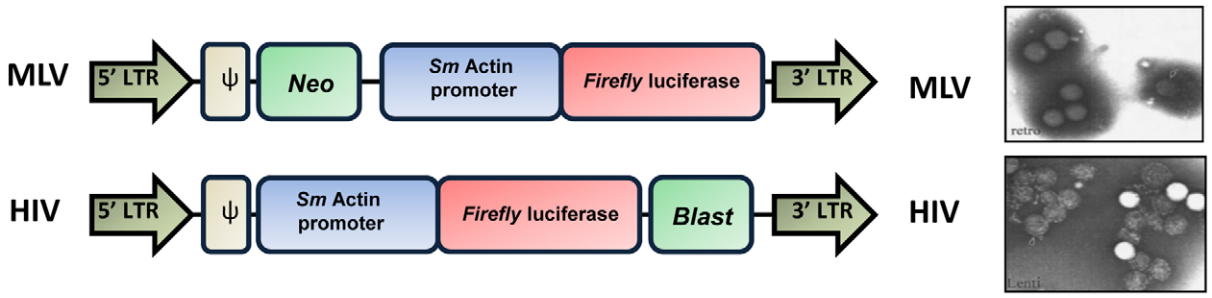


Figure 2. Representative pictures of the in vitro laid eggs (IVLE) collection, concentration, and in vitro development. (A) Female of *S. mansoni* releasing eggs one day after perfusion. The mesh of the netwell is evident. (B) Female surrounded by IVLE in the bottom of the well. (C) IVLE during the concentration process 48 hours after perfusion. (D–J) Representative images of an individual egg laid in vitro through the developmental process from day 1 (D), 2 (E), 3 (F), 4 (G), 5 (H), and 6 (I) to day 7 (J) after perfusion. (K) IVLE during the hatching process; arrows indicate empty eggshells. (L) Diagram of snail infection. (M) Cercariae released from snails infected with miracidia from IVLE, at 42 days after snail infection. Scale bars: (A–C,K,M), 200 μm; (D–J), 20 μm.
doi:10.1371/journal.pntd.0001230.g002



Retroviral construct	G418 cfu/ml	Blastcidin cfu/ml	qRT-PCR copies/ml	Titration ratio - copies/cfu	
				qRT-PCR: G418	qRT-PCR: Blasticidin
MLV_ActLuc	1×10^3		6.66×10^6	6660	
MLV_ActLuc	1×10^4		1.42×10^7	1420	
MLV_ActLuc	2×10^3		6.47×10^6	3235	
MLV_ActLuc	4×10^5		7.03×10^7	176	
HIV_ActLuc		1×10^2	9.89×10^5		9890
HIV_SLGFP		1×10^7	2.8×10^9		280
HIV_ActGFP		6×10^7	1.27×10^9		21

Figure 3. Correlating RNA titer with infectivity. Schematic diagram of two MLV and HIV representative constructs and corresponding micrographs of the particles (from Higashikawa et al. [25], with permission). To determine infectivity titers, NIH 3T3 or HT1080 cells were infected with qRT-PCR-titrated MLV retrovirus or qRT-PCR-titrated HIV, carrying neomycin (*Neo*) or blasticidin (*Blast*) resistance genes, respectively. Cells were selected in G418 or blasticidin for 10 days and resistant colonies were stained and counted.
doi:10.1371/journal.pntd.0001230.g003

[3]. For retroviruses, in particular MLV, with which we have some experience, increasing the titer of active virions is a sound way to improve prospects for productive transduction of target germ line cells. Given the progress with transgenesis of schistosomes with pseudotyped MLV [9–11,20,23,24], comments on virion production are included below.

Can virion production be optimized? There are several factors to consider for an optimal virus production, including producer (host) cell strain and culture conditions, cell density and vitality by the time of the DNA transfection, amount and quality of plasmid DNA used for transfection, and recovery of viral particles. Factors that reduce the retrovirus half-life also should be considered, i.e., storage of virions, freeze-and-thaw cycles, temperature, pH, and presence of serum [25]. The higher the viral titer, the better the prospects are for chromosomal insertion of the proviral transgene [26]. Although protocols to produce retrovirus in vitro optimize to improve titers, contamination of virions with defective particles is a frequent problem. Defective virions include particles without envelope, without RNA, or with RNA but non-infectious [27]. Using at least two approaches to estimate viral titer is recommended [25,27]. One approach should estimate the titer of infectious particles, i.e., a functional, biological assay where a cell line is infected with serial dilution of the virions and colonies of cells are selected by maintenance in antibiotic for which resistance is conferred by the retrovirus. Second, a quantitative approach to estimate the copy number of particles by qPCR should also be performed in parallel. With findings from the parallel assay, total

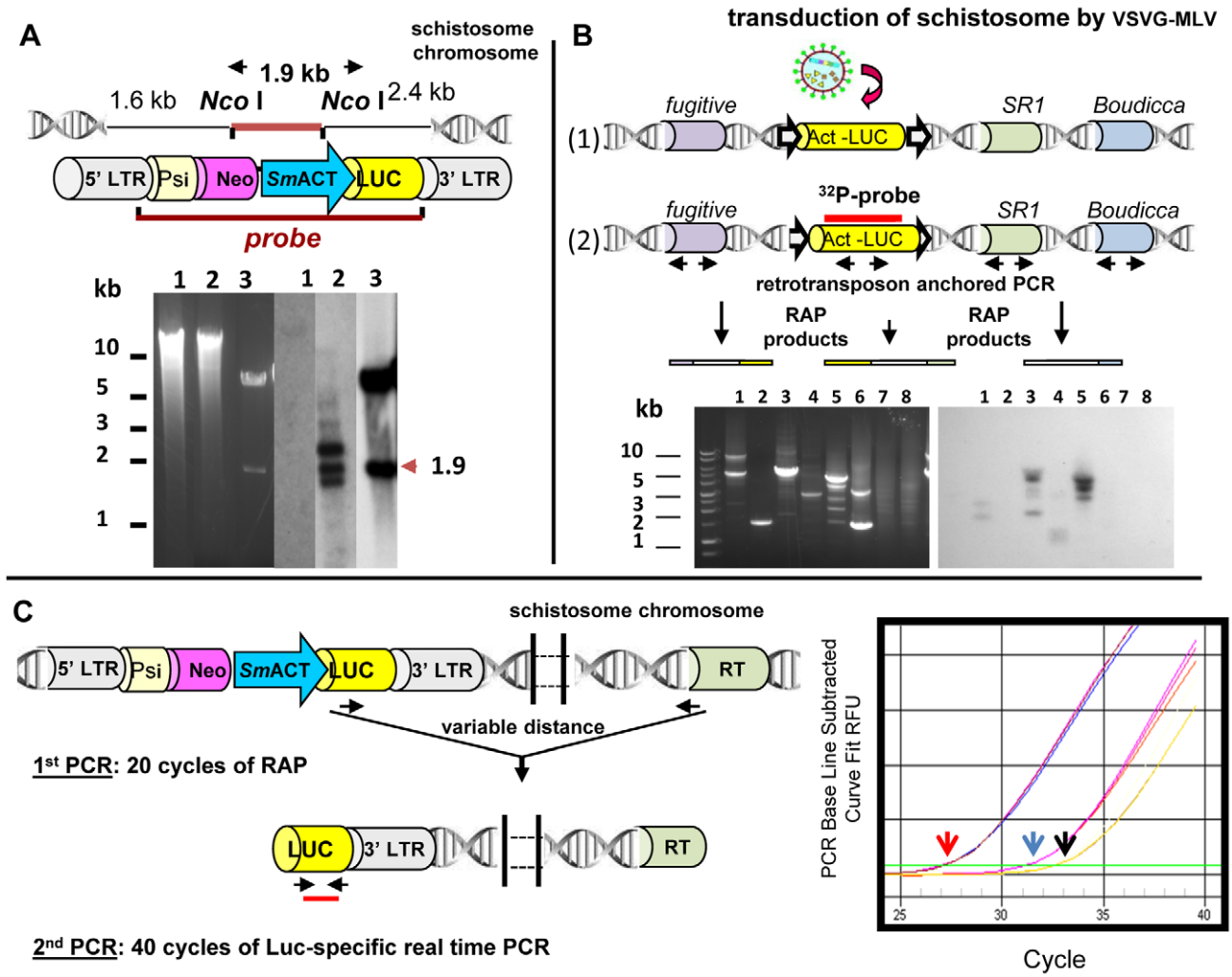


Figure 4. Approaches to identify, clone, and quantify integrated sequences into the schistosome genome. (A) Integration of retroviral provirus into the *S. mansoni* genome indicated by Southern hybridization analyses of genomic DNA from retrovirus-transduced schistosomes. *Top Panel*: Schematic representation of retroviral construct pLNHX-*Sm*ACT-Luc, showing the position of *Nco* I cleavage sites and also the location of the *Kpn* I fragment employed as the hybridization probe. The retrovirus cassette included the firefly luciferase reporter gene (yellow) driven by the *S. mansoni* actin 1.1 gene promoter (blue), and flanked by the 5′- and 3′-long terminal inverted repeats of the murine leukemia virus (grey). The cassette also included the gene endowing neomycin resistance (pink) and the psi motif (light yellow; involved in packaging the viral DNA). *Bottom Panel*: Southern hybridization analysis of genomic DNA from schistosomules transduced by VSVG-pseudotyped retrovirus. Left side: ethidium-stained gel of genomic DNAs of *S. mansoni*: lane 1, *Nco* I-digested gDNA from control, non-virus transduced cercariae; lane 2, *Nco* I-digested gDNA from schistosomules exposed to VSVG-pseudotyped pLNHX-*Sm*ACT-Luc virions; lane 3, *Nco* I-digested plasmid pLNHX-*Sm*ACT-Luc. Molecular size standards in kilobases (kb) shown at margin. Right side: autoradiograph of Southern hybridization signals from the *Nco* I-digested gDNAs and plasmid DNA from the left-side panel to the radiolabeled transgene probe (5.3 kb *Kpn* I fragment of pLNHX-*Sm*ACT-Luc, top panel). (Modified from [9] with permission.) (B) *Top Panel:* Schematic representation of the RAP technique, designed to recover integration junctions between integrated retroviral provirus and endogenous mobile genetic elements resident within the *S. mansoni* genome. (1) Schematic representation of the integration of the MLV retrovirus into schistosome chromosomes after transduction of cultured schistosomes by VSVG-pseudotyped MLV virions. Endogenous retrotransposons within the schistosome chromosomes are shown; numerous copies of *SR1*, *Boudicca*, and the *fugitive* have been described interspersed throughout the *S. mansoni* genome. (2) Schematic depiction of the RAP technique used to investigate transgene integrations. The position of the primers used in the PCRs and the probe used in Southern hybridizations is indicated. *Bottom Panel:* Left side. Ethidium-stained gels revealing RAP products amplified from gDNA extracted from schistosomula transduced with VSVG-pseudotyped pLNHX-SmACT-Luc virions; the PCR products were amplified using primers specific for the endogenous schistosome retrotransposons and the luciferase transgene. Right side: Southern hybridization of labeled retroviral transgene gene probe to the RAP products shown in top panel. Lane 1, RAP products amplified with luciferase left and *Boudicca* forward-directed primers; lane 2, RAP products from luciferase left- and *fugitive* forward-directed specific primers; lane 3, RAP products from luciferase left- and *fugitive* reverse-directed specific primers; lane 4, RAP products from luciferase left and *SR1* forward-specific primers; lane 5, RAP products from luciferase left- and *SR1* reverse-specific primers; lane 6, RAP products from luciferase left- and *SR2* forward-specific primers; lane 7, RAP products from luciferase left- and *SR2* reverse-specific primers; lane 8, RAP products from luciferase left- and SMα forward-specific primers. Values at left are molecular size standards (kb). (Modified from Kines et al. [9], with permission) (C) *Left Panel:* Illustration of the quantitative retrotransposon anchored PCR (qRAP). Schematic representation of the integration of the MLV retrovirus into schistosome chromosomes after transduction of cultured schistosomes by VSVG-pseudotyped MLV virions. First PCR: 20 cycles of end-point PCR preamplification with primers that target endogenous mobile genetic elements and luciferase transgene sequences. Heterogeneous amplicons of variable length are expected. Second PCR: quantitative PCR to estimate the copy number of luciferase-specific sequences within the transduced schistosome genome. Quantification was undertaken using copy number standards, i.e., 10-fold serial dilutions of the luciferase encoding plasmid pGL3, after which copy number of luciferase

transgene in schistosome genomic DNAs was calculated by interpolation from a standard curve. *Right Panel:* Amplification plots observed in MLV-transduced worms in preamplified template using *SR2* primer mix (red arrow), in preamplified template using only the luciferase transgene–specific primer as control of "one-way amplification" (blue arrow), and in non-preamplified template (black arrow). Arrows indicate the threshold cycle. RT: endogenous retrotransposon. (Modified from Rinaldi et al. [24], with permission.)
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number of virions particles and of intact, infectious particles can be established [25,27]. We use both approaches to estimate the viral titer and the ratio between copy number of particles and infectious units [24]. Figure 3 presents representative findings from our laboratory, for both MLV and HIV pseudotyped virions; in general, titers estimated by qPCR were two to three orders of magnitude greater than those estimated in biological assays. This is not only because qPCR is more sensitive, but also because qPCR detects non infective particles. Accordingly, the lower the titration ratio, the more efficient is the virus production in terms of viable infectious particles.

How Can We Analyze Integration of Transgenes into the Genome?

Integration is a pivotal step in establishing transgenic schistosomes. To confirm that vectors can integrate into schistosome DNA, methods to determine and extract integration junctions have to be employed. Integration junctions cannot be obtained simply by regular PCR procedures because the genomic flanking sequences are unknown. Southern hybridization employing informative restriction enzymes and probes retain a key position in these studies. For example, Figure 4A presents a Southern hybridization analysis to confirm the presence of proviral transgenes in the genome of schistosomules exposed to pseudotyped MLV virions. However, the definitive proof of integration of the transgenes into the schistosome chromosome requires the use of PCR-based approaches directed at cloning and sequencing the integration junction (Figure 4B). Given that draft genomes of *S. mansoni* and *S. japonicum* are available, BLAST analysis of cloned sequences flanking the transgenes (e.g., MLV retrovirus) can readily verify that the transgenes have integrated into a schistosome chromosome. A number of PCR-based methods can be employed to recover integration events and unknown host genome sequences flanking the transgene. These include inverse PCR, linker ligation PCR and thermal asymmetric interlaced (TAIL)-PCR, and *Alu*-PCR [28,29]. We developed an *Alu*-PCR-like approach termed retrotransposon anchored PCR (RAP) [8,9], which relies on anchoring primers to multicopy endogenous mobile genetic elements interspersed in the schistosome genome to locate integration junctions of transgenes in the genome of *S. mansoni* (Figure 4B). Furthermore, we have adapted RAP for quantitative PCR in order to determine comparatively the number of MLV transgenes within the genome of transduced populations of schistosomes (Figure 4C) [24]. In addition, very high-throughput sequencing using Illumina technology could be utilized, as has been demonstrated for transposon-based insertional mutagenesis of *Salmonella* Typhi [3]. This latter approach could determine the exact location of transgenes within the schistosome genome.

Outlook

As noted, transgenic schistosomes have been created (e.g., [7–9,23]). However, improvements are needed—and certain to take place—in order to establish transgenic schistosomes and protocols. A crucial impediment to date has been the difficulty of delivering transgenes to chromosomes of the germ line. Targeting integration-competent vectors to IVLE may surmount this limitation. Moreover, there are other points in the developmental cycle where the germ line might be accessed, including the daughter sporocysts where the germ cells are comparatively large (Coustau and Yoshino [30] and references therein). We can look forward to advances in technologies that 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 *Fasciola* and *Opisthorchis* species, and to neglected helminth parasites at large.

Supporting Information

Alternative Language Abstract S1 Translation of Abstract into Thai by Sutas Suttiprapa.
(PDF)

Alternative Language Abstract S2 Translation of Abstract into Spanish by Gabriel Rinaldi.
(PDF)

Key Learning Points

- Draft genome sequences for *S. mansoni* and *S. japonicum* are now available. Accordingly, there is a pressing need now to develop functional genomics tools for schistosomes to determine the importance of these new genes.
- Functional genomics approaches hold promise to determine the nature and importance of genes of the human schistosomes.
- Retroviral and transposon-mediated gene manipulation, using integration-competent, vector-based technologies, have been shown to be feasible for schistosomes.
- The retrovirus murine leukemia virus, which is widely used in stem cell and gene therapy research, and the *piggyBac* transposon, originally isolated from the genome of a moth, have now been shown to be active in schistosomes, to integrate into schistosome chromosomes, and to provide gains-of-function for the reporter genes firefly luciferase, GFP, and neoR.
- Both MLV and *piggyBac* both have potential in high-throughput insertional mutagenesis studies, feasible now that draft genome sequences are available.
- Vector-based RNAi—retroviral vector-mediated RNA interference demonstrated in schistosomes—targeting a hemoglobin-digesting protease provides proof-of-principle that vector-based RNAi is feasible to target any of the ~13,000 protein encoding genes of the schistosome.

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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 ~13 000 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.

Key words: 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 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*.

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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 supercoiled 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

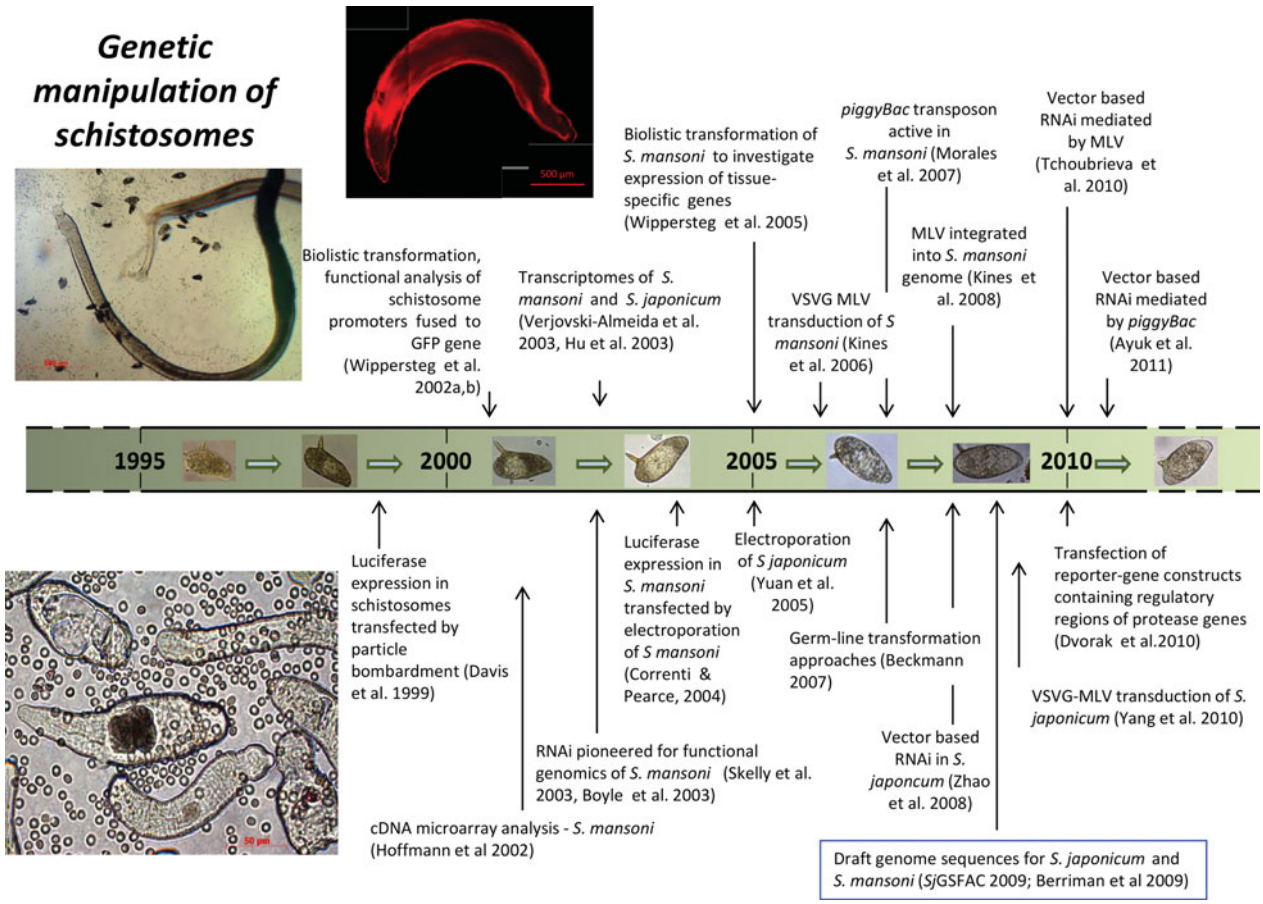

Fig. 1. Schematic time line of advances on genetic manipulation of schistosomes. Key events are noted, with pioneering or key reports cited.

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 virion-transduced 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-BASED RNAi

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

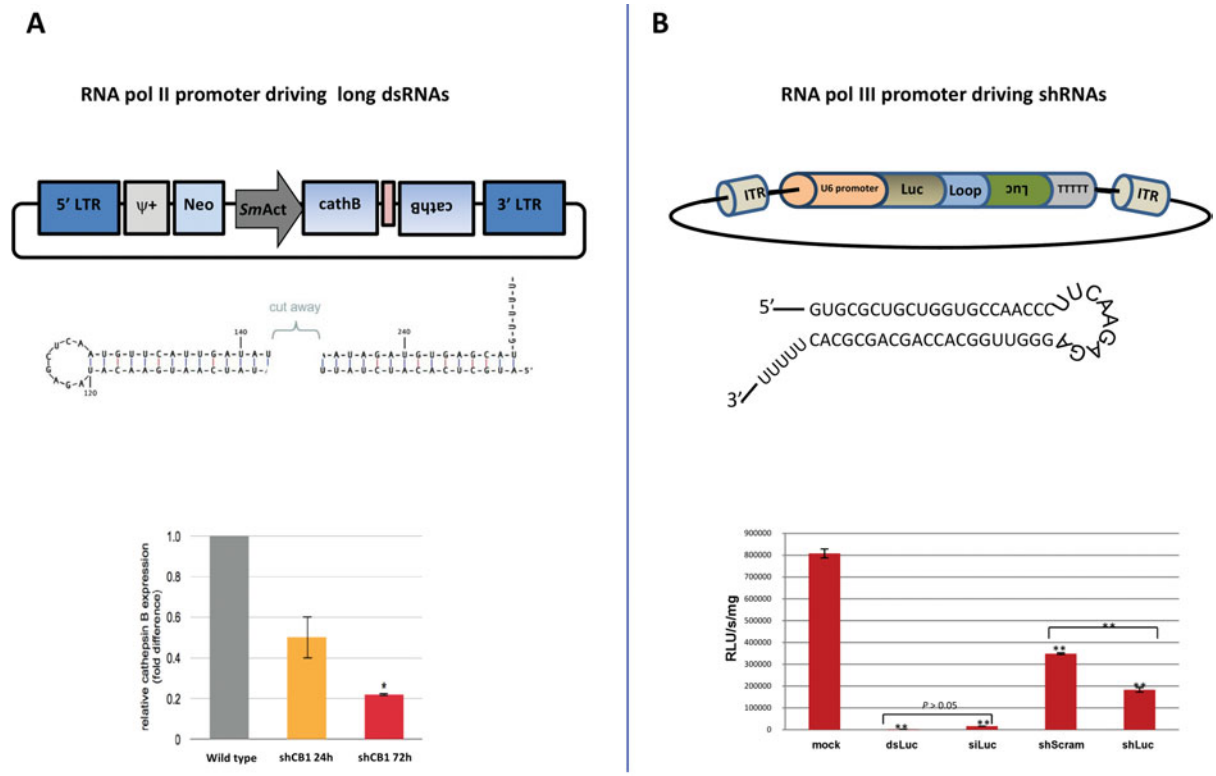

Fig. 2. Vector-based RNA interference (RNAi) in *Schistosoma mansoni*. Panel A: Vector-based RNAi mediated by long hairpin RNAs (hpRNA) driven by a RNA polymerase II promoter (from the *S. mansoni* actin gene) carried by a retroviral vector to knock down of the *S. mansoni* cathepsin B1. Top: schematic 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.

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 tapeworms 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

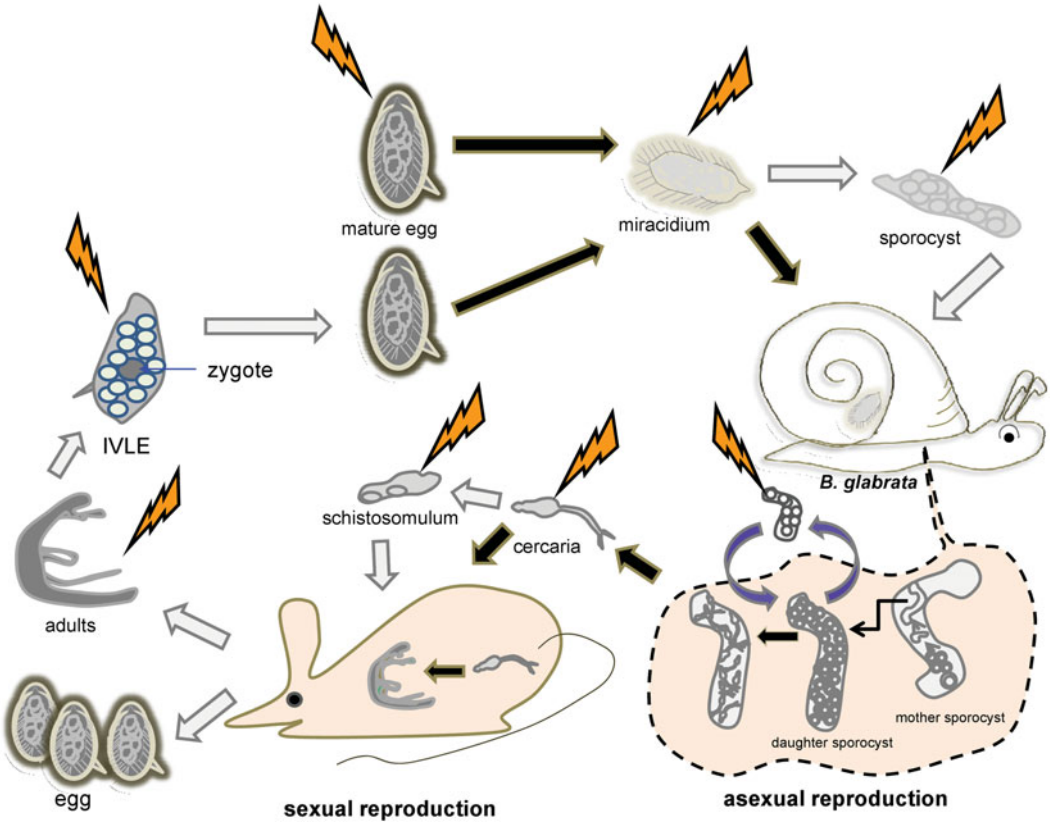

Fig. 3. Cartoon representation of points of the developmental cycle of *Schistosoma mansoni* amenable to genetic manipulation. Both the mammalian stages involved in sexual reproduction and the snail stages with asexual reproduction are presented. Thunder bolts suggest accessible points of introduction 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.

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.

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