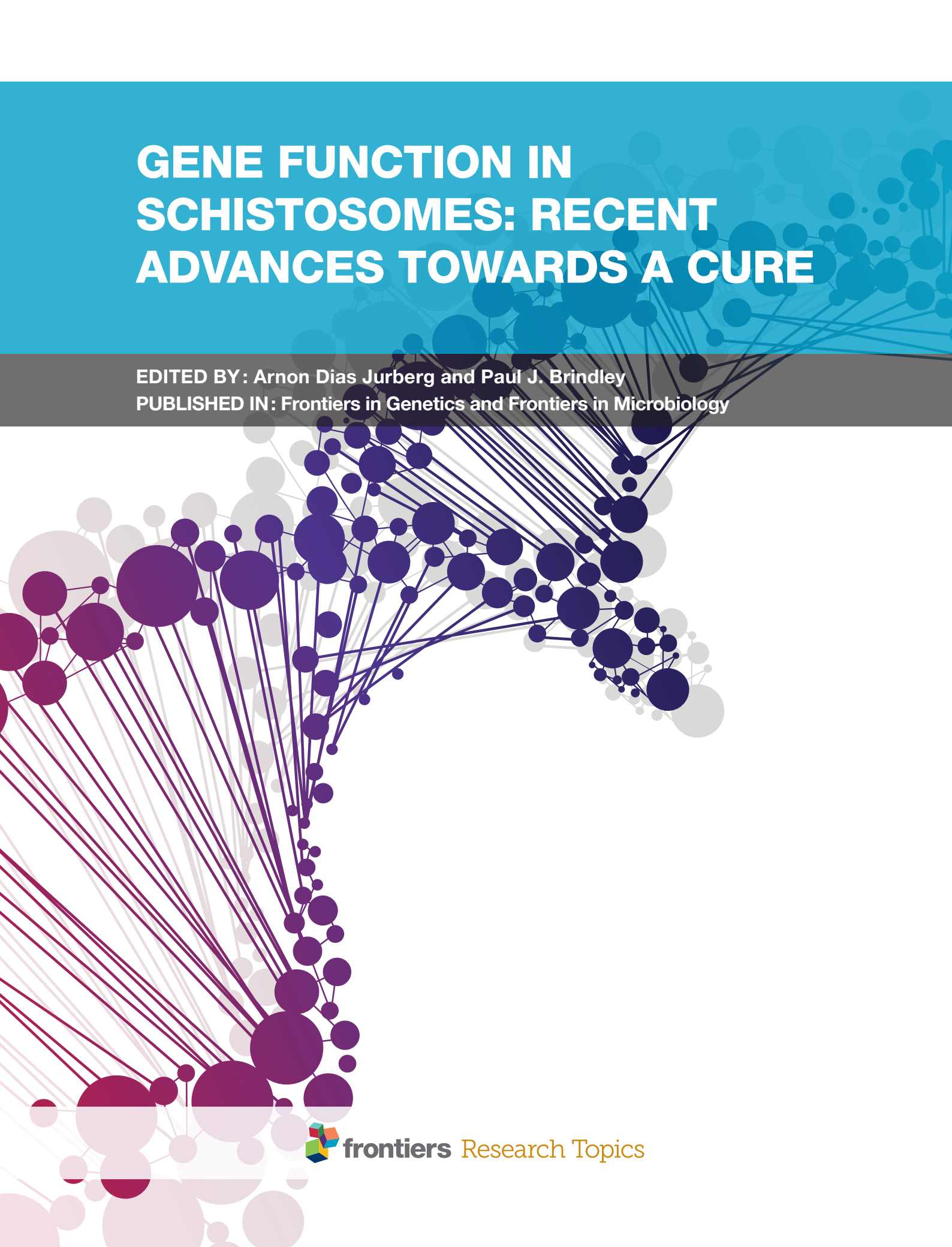
GENE FUNCTION IN SCHISTOSOMES: RECENT ADVANCES TOWARDS A CURE

EDITED BY: Arnon Dias Jurberg and Paul J. Brindley
PUBLISHED IN: Frontiers in Genetics and Frontiers in Microbiology



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ISSN 1664-8714
ISBN 978-2-88919-556-5
DOI 10.3389/978-2-88919-556-5

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GENE FUNCTION IN SCHISTOSOMES: RECENT ADVANCES TOWARDS A CURE

Topic Editors:
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Representative endemic regions for schistosomiasis, in this example in rural Minas Gerais and São Paulo states, Brazil. The triptych illustrates the role of sub-standard sanitation, human activities in contaminated watercourses, which also are ecosystems that include the snail intermediate hosts of schistosomiasis (photographs kindly provided by Paulo Marcos Zech Coelho and Áureo Almeida, René Rachou Research Center/Fiocruz-MG, Brazil).

Schistosomes are human parasites distributed worldwide in tropical and sub-tropical latitudes, especially in developing countries and impoverished regions. These neglected tropical disease (NTD) pathogens causes debilitating illnesses, which include hepatosplenomegaly, hepatic fibrosis, haemorrhagic necrotic ulcerations in the intestinal mucosa, urogenital tract diseases, in addition to cardiopulmonary, renal and neurologic lesions due to egg accumulation in the liver, intestines, uro-genital tissues and other sites. Urogenital schistosomiasis is a risk factor for bladder cancer and increases the risk of transmission of HIV infection.

Despite extensive effort to control this NTD over the years, deployment on a considerable scale of commercially available drugs in endemic populations has induced the emergence of resistant isolates and raised the need to identify new targets for alternative therapies. Because of the availability of genomes of the three major species of human schistosomiasis, and through advances in functional genomics and live imaging, studies on schistosomes have now come into focus as models to investigate adaptations to parasitism and developmental biology of trematodes and cestodes, and indeed flatworms and Lophotrochozoans, at large.

This Research Topic aims at gathering state-of-art essays on schistosome genetics, genetics, pathobiology and immunobiology. It also aims to highlight advances in understanding of the host-parasite relationship, in paradigms that address this NTD, and to discuss new perspectives and advances in chemotherapy and immunoprophylaxis.

Citation: Jurberg, A. D., Brindley P. J., eds. (2015). Gene Function in Schistosomes: Recent Advances Towards a Cure. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-556-5

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frontiers in Genetics

EDITORIAL
published: 15 April 2015
doi: 10.3389/fgene.2015.00144

Gene function in schistosomes: recent advances toward a cure

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Keywords: schistosomiasis, economical impact, poverty, treatment, gene function

Neglected Tropical Diseases (NTDs) drive poverty and social inequality; indeed NTDs are manifestations of these problems (Bardosh, 2014). Poverty is a major detriment to human development. Measuring poverty by estimating poverty lines has led the World Bank to initially set the income value of US$1 a day and later update it to US$1.25 a day at 2005 purchasing power parity as the revised international poverty line (Ravallion et al., 1991, 2009). This meant that in 2005 people earning less than US$37.5 a month lived in poverty—about 1.4 billion people worldwide (Chen and Ravallion, 2008). Despite considerable efforts and highly uneven temporal and regional improvements since that point (Chen and Ravallion, 2008; United Nations, 2013), each one of these impoverished persons is potentially exposed to infectious diseases due to precarious conditions, such as contaminated drinking water, malnutrition, lack of sanitation or shelter, and inadequate basic services including education, health and security (WHO-World Health Organization, 2012; United Nations, 2013).

However, because they mostly impact populations of lower-income countries, the NTDs receive relatively little attention and funding from the international community. For example, whereas helminth infections are estimated to affect more than one billion people in sub-Saharan Africa, Asia and the Americas (Hotez et al., 2007), the global expenditures on helminth infection targeted research and development (R&D) was a mere US$66.8 million in 2008, with uneven distribution across diverse diseases and focus mainly for basic research (Institute of Medicine, 2011). In comparison, Kanavos et al. (2010) have estimated a global investment of more than US$17 billion on cancer R&D in 2007, with estimates for 2008 of more than 12.7 million new cases and ∼7.6 million deaths (Ferlay et al., 2010). Although, deaths due to helminth infection, estimated to be around 300,000 deaths a year, are not as imposing as for some other infectious diseases associated mortality, helminth infection-induced burden of disease results in premature and ongoing disability and impediments throughout life, and impacts negatively and largely on the economy of endemic regions (Hotez et al., 2006). Hence, these characteristics led to the grouping of 17 parasitic, bacterial and viral diseases as NTDs by the World Health Organization (Salaam-Blyther, 2014).

Schistosomiasis is one of these NTDs, and moreover is considered the most important of the helminth diseases in terms of morbidity and mortality (Hotez et al., 2008; King, 2015). Among the most problematic of the NTDs due to its extensive geographical distribution and public health impact, schistosomiasis is caused by infection with trematode worms of the genus *Schistosoma*. Infection occurs in contaminated freshwater through skin penetration of a larval form of the worm known as the cercaria. Cercariae are produced by clonal expansion of germinal cells in the intermediate snail host following infection of the snail by a ciliated larva termed the miracidium (Pan, 1965; Cheng and Bier, 1972; Jurberg et al., 2008). There are species-specific, geographical constraints among the schistosome and the intermediate host. *Schistosoma mansoni* infects snails of the genus *Biomphalaria*, whereas *S. japonicum* and *S. haematobium* infect the genera *Oncomelania* and *Bulinus*, respectively. In these snails, the invading miracidium first

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Genetics

Received: *16 February 2015*
Accepted: *26 March 2015*
Published: *15 April 2015*

Citation:
Jurberg AD and Brindley PJ (2015) Gene function in schistosomes: recent advances toward a cure. Front. Genet. 6:144. doi: 10.3389/fgene.2015.00144

transforms into mother-sporocyst then into daughter-sporocysts prior to generation of cercariae, which escape from the snail back to the freshwater. Cercariae actively swim toward a human host where they penetrate the skin directly (Pan, 1965). Within the human skin, the cercaria sheds its tail, transforms into a blood vessel inhabiting form termed the schistosomulum, which develops within the bloodstream or to a lesser extent the lymphatic system (Miller and Wilson, 1978). These schistosomula circulate with the blood as they develop to sexually mature blood flukes, sometimes completing two or three systemic circuits until they establish residency in the portal hepatic tract (*S. mansoni* and *S. japonicum*) or the pelvic organs (*S. haematobium*) (Wilson, 2009). Unlike other platyhelminths, schistosomes evolved dioecism—separate sexes—and need to mate for producing eggs. Eggs are released into the bloodstream and many embolize in smaller blood vessels and capillaries of diverse organs, inducing the characteristic granulomatous reaction of schistosomiasis (see Hams et al., 2013). Eggs reaching the intestines (*S. mansoni* and *S. japonicum*) or the bladder (*S. haematobium*) induce inflammatory responses that facilitate passage of the schistosome egg through the wall of bowel or bladder, respectively, to the lumen from where the eggs exit to the external environment in feces or urine (Lenzi et al., 1987 for *S. mansoni*). On the reaching freshwater, the egg hatches to release the motile, ciliated miracidium, thereby completing the developmental cycle of the pathogen, as depicted in **Figure 1**. Eggs that fail to be eliminated may cause different symptoms depending on the schistosome species, but frequently cause chronic inflammation and fibrosis (Gryseels et al., 2006; Hams et al., 2013; Colley and Secor, 2014).

In spite of the information on schistosome biology at present, with 14,933 published papers from 1980 to 2014 (using "*Schistosoma*" as the query at PubMed)—which accounts for 78% of all the retrieved schistosome literature at this database—a definitive cure for schistosomiasis still faces daunting challenges and may be far from being achieved. In particular, 71% of the research funds for schistosomiasis in 2008 (approximately US$14 million) were designed to basic instead of applied investigation (Institute of Medicine, 2011). Yet, concurrent presence of schistosomes at different stages of development floods the infected person with a battery of diverse antigens, which induce conflicting immune responses (Colley and Secor, 2014). Moreover, naturally occurring re-infections raise the possibility that immunization against schistosomiasis may not work (Colley and Secor, 2014). Another aspect of controlling schistosome infection regards education and sanitation, once informed endemic populations still insist on, and/or frequently have no alternative but using the contaminated watercourses for bathing, laundry and other household activities, recreation, and so forth (Enk et al., 2010) (**Supplementary Figure 1**). Indeed, the sustained and repeated use of praziquantel in endemic regions raises the justifiable worry for the appearance of drug resistance (Cioli et al., 2014). To raise hopes for fighting schistosomiasis, game-changing advances and tools from other fields of research are being adapted and implemented for research on schistosomes, especially the strategies related to the study of gene function (Hoffmann et al., 2014). For example, deployment of the clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas system for genome editing (see Doudna and Charpentier, 2014) and/or schistosome transgenesis for gain-of-function manipulation (Mann et al., 2014) extends the range of experimental approaches to interrogate the host-parasite relationship and to test novel vaccines and other interventions. It is now the time for research on schistosomiasis to evolve from -omics to function.

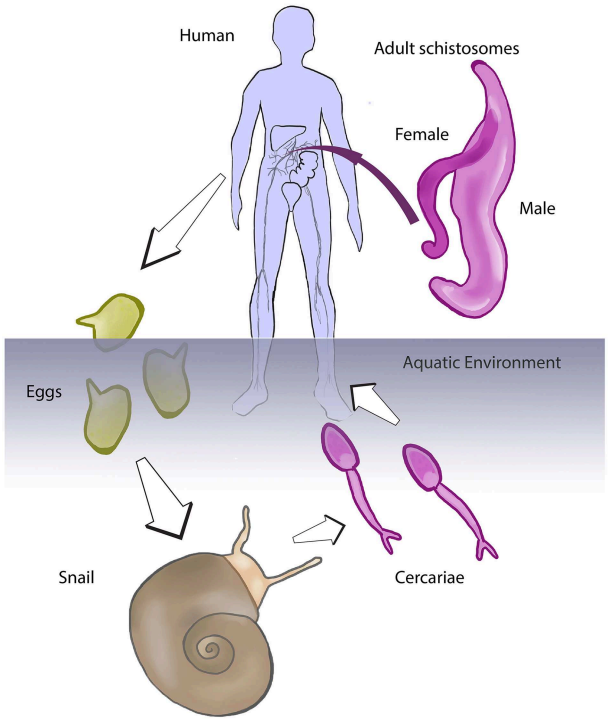

FIGURE 1 | Developmental cycle of the schistosome. Schistosomes are obligate parasites that evolved a complex developmental cycle using two hosts, a snail and a human (or other mammal). In the human (or laboratory mouse, or other permissive mammalian host), the worms reside within the blood and reproduce sexually. Eggs released by the female schistosome exit to the external environment with the feces or urine. The egg hatches in freshwater, releasing the miracidium larva that infected the aquatic snail. The parasite undergoes asexual reproduction in the snail, producing cercariae that are shed from the snail into the water. Cercariae penetrate the skin of the human in contact with contaminated water, to complete the developmental cycle of the pathogen (we acknowledge Figure 1 of Gryseels et al. (2006) for inspiration for this schematic).

Our new research topic includes 15 papers, both primary research articles and reviews from a representative cadre of the leading experts in the field of research on schistosomes and schistosomiasis, and neglected tropical diseases. The themes span molecular genetics including chromosomal evolution, epigenetic control of schistosome genes, vaccine studies including targeting proteolysis and enzyme inhibitors central to the physiology of the parasites, and how infection with this NTD pathogen induces bladder cancer. The reports also address signaling pathways, including insulin receptors in these pathogens, kinomes, and kinases, glycogenome, molecular studies on sex differentiation

and host-parasite interactions including the snail-schistosome relationship. The 91 authors are from 14 countries, including from regions where schistosomiasis is endemic such as Angola, Egypt, Brazil, and China. We hope you will be informed by this series, as well as enjoy the authors' scholarly contributions, that the work and ideas presented advance the field toward better control or even a cure for schistosomiasis, and that this Research Topic elevates investigation in schistosomiasis and related NTDs into the more newsworthy areas of emerging and infectious disease research.

Acknowledgments

We thank Paulo Marcos Zech Coelho and Áureo Almeida for kindly providing the photographs and Meredith E. Brindley for expert assistance with the artwork and figures. AJ received support from awards 400408/2013-9, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and E-26/111.086/2014, Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ). PB received support from awards CA155297 and CA164719, National Cancer Institute, US National Institutes of Health.

Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fgene.2015.00144/full

Supplementary Figure 1 | A representative endemic region for schistosomiasis, in this example in rural Minas Gerais state, Brazil. The triptych illustrates the role of sub-standard sanitation and of human activities in contaminated watercourses that also are ecosystems that include the snail intermediate hosts of schistosomiasis (Photographs kindly provided by Áureo Almeida and Paulo Marcos Coelho, René Rachou Research Center/Fiocruz-MG, Brazil).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in GENETICS

MINI REVIEW ARTICLE
published: 23 December 2014
doi: 10.3389/fgene.2014.00444

Schistosome and liver fluke derived catechol-estrogens and helminth associated cancers

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Infection with helminth parasites remains a persistent public health problem in developing countries. Three of these pathogens, the liver flukes *Clonorchis sinensis*, *Opisthorchis viverrini* and the blood fluke *Schistosoma haematobium,* are of particular concern due to their classification as Group 1 carcinogens: infection with these worms is carcinogenic. Using liquid chromatography-mass spectrometry (LC-MS/MS) approaches, we identified steroid hormone like (e.g., oxysterol-like, catechol estrogen quinone-like, etc.) metabolites and related DNA-adducts, apparently of parasite origin, in developmental stages including eggs of *S. haematobium*, in urine of people with urogenital schistosomiasis, and in the adult stage of *O. viverrini*. Since these kinds of sterol derivatives are metabolized to active quinones that can modify DNA, which in other contexts can lead to breast and other cancers, helminth parasite associated sterols might induce tumor-like phenotypes in the target cells susceptible to helminth parasite associated cancers, i.e., urothelial cells of the bladder in the case of urogenital schistosomiasis and the bile duct epithelia or cholangiocytes, in the case of *O. viverrini* and *C. sinensis*. Indeed we postulate that helminth induced cancers originate from parasite estrogen-host epithelial/urothelial cell chromosomal DNA adducts, and here we review recent findings that support this conjecture.

Keywords: urogenital schistosomiasis, opisthorchiasis, catechol-estrogens, oxysterols, DNA-adducts, neglected tropical disease-associated-cancer, squamous cell carcinoma of the bladder, cholangiocarcinoma

BIOLOGICAL CARCINOGENS – THREE HELMINTH PARASITES

The World Health Organization's International Agency for Research on Cancer (IARC) and the United States' National Institutes of Health (NIH) consider that ∼20% of cancers are caused by infectious diseases. Some cancer-inducing infectious agents, such as Hepatitis B and C Viruses, are well known. However, less appreciated are the several major human helminth pathogens that cause cancer. IARC recognizes three helminth infections as definitive causes of cancer – the fish-borne liver flukes *Opisthorchis viverrini* and *Clonorchis sinensis* and the blood fluke *Schistosoma haematobium* (Bouvard et al., 2009; de Martel et al., 2012; International Agency for Research on Cancer (IARC), 2012; **Figure 1**). In addition to direct detriment on development and health of infected populations, infection with liver flukes and schistosomes – types of helminth parasites collectively termed trematode flatworms – lead to infection related cancers, specifically cholangiocarcinoma (CCA; bile duct cancer) and squamous cell carcinoma (SSC) of the urinary bladder, respectively.

UROGENITAL SCHISTOSOMIASIS AND BLADDER CANCER

Three major species of schistosomes are the agents of human schistosomiasis – *Schistosoma japonicum* and *Schistosoma mansoni* cause intestinal schistosomiasis in East Asia, Africa, South America, and the Caribbean while *S. haematobium*, occurring widely through Africa and the Middle East, causes urogenital schistosomiasis (**Figure 1**). In the range of 4.5–70 million disability adjusted life years (DALYs) are lost to schistosomiasis (King and Dangerfield-Cha, 2008). More people are infected with *S. haematobium* than with the other schistosomes. Of ∼112 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 kidney damage (van der Werf et al., 2003; Hotez et al., 2009; King, 2010). In many patients, deposition of *S. haematobium* parasite ova eventually leads to SSC of the bladder (Hodder et al., 2000; Parkin, 2006). Moreover, as many as 75% of women infected with *S. haematobium* suffer from female genital schistosomiasis (FGS) of the lower genital tract (Hotez et al., 2009). FGS results from

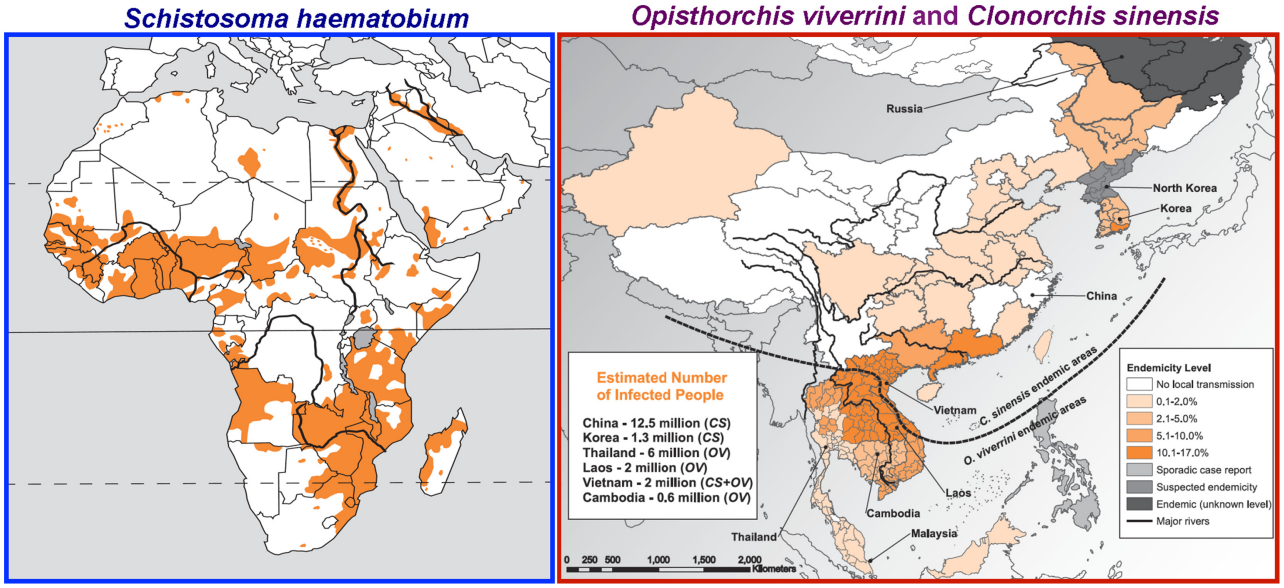

FIGURE 1 | Geographical distribution of the carcinogenic flukes. Left: Distribution of the blood fluke *Schistosoma haematobium* (sub-Saharan Africa, Nile valley in Egypt and Sudan, the Maghreb, and the Arabian peninsula). Right: Distribution of the liver flukes *Clonorchis sinensis* (China, Republic of Korea, Democratic People's Republic of Korea, the Russian Federation, and northern Viet Nam) and *Opisthorchis viverrini* (Thailand, Laos, Cambodia, Malaysia, and southern part of Viet Nam). "No local transmission" stands for "no reported local transmission." From International Agency for Research on Cancer (IARC), (2012); permission requested from WHO Press, International Agency for Research on Cancer.

deposition of schistosome eggs in the uterus, cervix, vagina and vulva, with ensuing inflammatory responses. It impairs fertility (Santos et al., 2014) and also increases susceptibility of the woman to HIV (Feldmeier et al., 1994; Kjetland et al., 2006; Ndhlovu et al., 2007; Jourdan et al., 2011).

Squamous cell carcinoma is a malignant, poorly differentiated neuroendocrine neoplasm. SCC is the common form of bladder cancer in rural Africa where *S. haematobium* is prevalent (Mostafa et al., 1999; Zhong et al., 2013). In contrast, the majority of bladder cancer in developing countries and regions not endemic for urogenital schistosomiasis is transitional cell carcinoma (TCC) that arises from the transitional epithelium lining of the bladder. The parasite eggs trapped in the bladder wall release antigens and other metabolites (presumably evolved to expedite egress to the urine, and hence to the external environment). Nonetheless, the phenomenon leads to hematuria and to chronic inflammation, in turn increasing risk of urothelial hyperplasia, dysplasia, and SCC of the bladder (Honeycutt et al., 2014). The epidemiologic association between SSC of the bladder with schistosomiasis haematobia is based both on case control studies and on the correlation of bladder cancer incidence with prevalence of infection with *S. haematobium* within different geographic locations. Schistosomiasis haematobia is a chronic infection, the adult, egg-producing schistosomes live for many years, re-infections frequently occur, and schistosomiasis associated bladder SCC appears relatively early, often by the mid-decades of life. By contrast, TCC usually presents in the later decades of life. The incidence of urogenital schistosomiasis associated SCC is estimated in 3–4 cases per 100,000 (Shiff et al., 2006).

FISH-BORNE FLUKES AND BILE DUCT CANCER

Liver infection caused by *O. viverrini*, *C. sinensis* and related flukes remains a major public health problem in East Asia and Eastern Europe where >40 million people are infected. *O. viverrini* is endemic in Thailand, Lao PDR, Vietnam and Cambodia (Sripa et al., 2011; Sithithaworn et al., 2012; **Figure 1**). Humans acquire the infection with *O. viverrini* by eating undercooked, fresh water cyprinoid fish infected with the metacercariae of the fluke (Sripa et al., 2011). There the parasites mature over 6 weeks into adult flukes, which graze on biliary epithelia. Eggs of *O. viverrini* are shed in bile and exit the infected person with the fecal stream. Freshwater snails ingest the eggs; the parasite (and related flukes, above) undergoes transformations within the snail host, culminating in the release of cercariae that seek out and penetrate the skin of a freshwater fish. Where sanitation is less than optimal, eggs may enter fresh water ecosystems where the eggs are ingested by freshwater snails. Human infection leads to hepatobiliary disease, cholangitis, obstructive jaundice, hepatomegaly, periductal fibrosis, cholecystitis, and cholelithiasis (Blechacz et al., 2011; Mairiang et al., 2012). More problematically, experimental and epidemiological evidence implicates liver fluke in the etiology of a major sub-type of liver cancer, CCA or bile duct cancer [Bouvard et al., 2009; de Martel et al., 2012; International Agency for Research on Cancer (IARC), 2012].

Cholangiocarcinoma, bile duct cancer, is an adenocarcinoma of the bile ducts, with a dismal prognosis. These are slow growing tumors, which spread along bile ducts with periductal and mass forming extensions. Prognosis is poor owing to the silent clinical character, difficulty in early diagnosis, and limited therapeutic approaches, especially in resource poor settings such

as northeastern Thailand where the recent estimate of median survival time after supportive treatment was 4 months (Thunyaharn et al., 2013). Surgical management is the only potentially curative treatment, but is restricted to early-stage disease. CCA has a worldwide distribution, beyond East Asia, where patients often develop CCA *de novo* without obvious risk factors. Primary sclerosing cholangitis and congenital bile duct anomalies are also precursors. In Thailand and elsewhere in East Asia, where infections with liver flukes are definitive risk factor, the factors share a common determinant of chronic inflammation and chronic injury of the biliary epithelium, including from persistent parasitism by these fish-borne trematodes (Sripa et al., 2009, 2012; Blechacz et al., 2011; Johnson et al., 2012; O'Hara et al., 2013; Razumilava and Gores, 2013).

FLUKES, CATECHOL-ESTROGENS, OXYSTEROLS, AND CARCINOGENESIS

In addition to the hormone-like effects of the parasite estradiol-related molecules on the endocrine and immune system of the host, initiation metabolites of estrogens can be also considered as carcinogenic chemicals (Cavalieri and Rogan, 2011, 2014). Hydroxylation of estrogens forms the 2- and 4-catechol estrogens involved in further oxidation to semiquinones and quinones, including formation of the catechol estrogen-3, 4-quinones, the major carcinogenic metabolites of estrogens. These electrophilic compounds react with macromolecules, including DNA, to form the depurinating adducts that eventually lead to mutations and cancer initiation (**Figure 2**; Cavalieri and Rogan, 2011). Several mechanisms explain the role of estrogens in disease. The better-known hypothesis is that the estrogen receptor mediates cell proliferation, increasing errors in DNA replication (Clemons and Goss, 2001; Yager and Davidson, 2006; Botelho et al., 2009b, 2013). Another interpretation postulates that estrogen metabolites react covalently with DNA bases by redox cycling or by forming an abasic site. Subsequent error-prone repair of the modified DNA generates oncogenic mutations that initiate cancer. The two mechanisms may act in concert. According to the second mechanism, P450 metabolism of estrone and estradiol generates the catechol estrogens, 2-hydroxyestrogen and 4-hydroxyestrogen. Further oxidation leads to 2, 3-catechol-

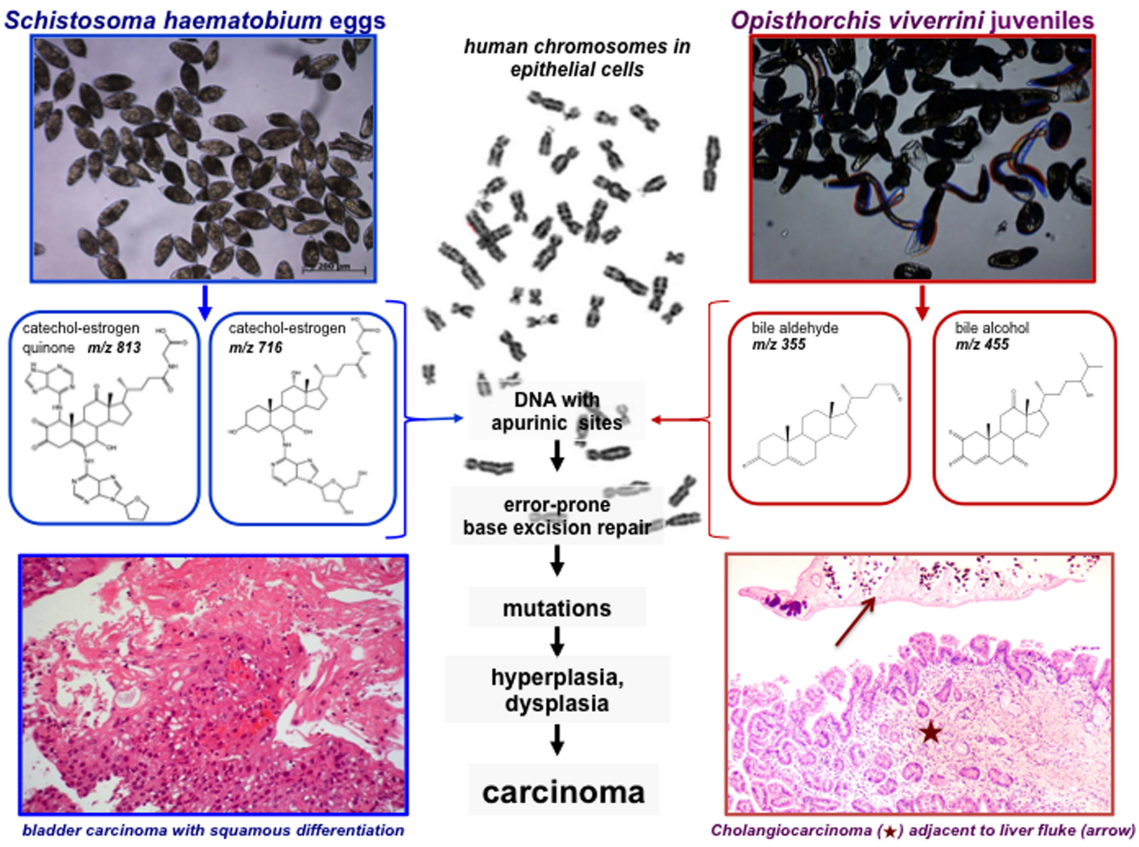

FIGURE 2 | Carcinogenesis mediated by steroid hormone like molecules derived from *S. haematobium* and *O. viverrini*. Eggs of *S. haematobium* derived catechol-estrogens and DNA-adducts (left; Botelho et al., 2013), and *O. viverrini* derived oxysterols (right; Vale et al., 2013), likely interact with the chromosomes of target cells inducing DNA apurinic sites that eventually escape the DNA repair mechanisms leading to mutations. These mutations ultimately would transform the target cell, leading to hyperplasia and ultimately to neoplasia, i.e., squamous cell carcinoma of the bladder [bottom left, brief description adapted from Santos et al. (2014)] and liver fluke induced cholangiocarcinoma (CCA; bottom right). Hematoxylin and eosin stained section of human intrahepatic bile duct/liver, cholangiocarcinoma at bottom of image (star) and an adult *O. viverrini* liver fluke (arrow) at top. Image contributed by co-author Banchob Sripa. Human metaphase chromosomes – image from Tang et al. (2013).

estrogen quinine and 3, 4-catechol-estrogen quinone, respectively, which can react directly with DNA via a Michael addition or indirectly via generation of reactive oxygen species. Methylation of catechol estrogens by catechol-*O*-methyltransferase, conjugation of the catechol estrogen quinones with glutathione, and enzymatic reduction to reform catechol estrogens are processes that prevent accumulation of the highly reactive metabolites. However, if the latter protective processes are insufficient, catechol estrogen quinones accumulate, which damage DNA either by oxidation or depurination, and release of catechol estrogen modified purines (Liehr et al., 1986; Cavalieri et al., 1997; Yager and Davidson, 2006).

While examining human cases of urogenital schistosomiasis from Angola, we observed elevation in levels of estradiol in sera but not luteinizing hormone (LH) or testosterone (Botelho et al., 2009a). Estradiol is a steroid hormone secreted principally by the ovarian follicles in vertebrates. It seemed implausible that the elevated levels could be attributed to hypothalamic–pituitary–gonadal axis regulation. Rather, we speculated that schistosomes produced the estradiol-related metabolites that contributed to the elevated estradiol levels. Using mass spectrometry approaches (Gouveia et al., 2013) we characterized >20 estradiol related metabolites, from sera of *S. haematobium*-infected persons from Angola and, remarkably, in the parasites including the eggs (Botelho et al., 2013). Catechol-estrogens are formed by hydroxylation on the steroid aromatic ring A. Hydroxylation of both C-2 and C-3 on a steroid ring was apparent and, further, oxidation to an estradiol-2,3-quinone. The schistosome estrogenic metabolites readily seen in urine and *in vitro* appear to arise by reactions of quinones of catechol estrogens with chromosomal DNA (Botelho et al., 2011b, 2013). In addition, we exposed non-cancerous CHO (Chinese hamster ovary) cells to secretions and lysates of *S. haematobium eggs* and adult parasites, which stimulated cellular proliferation, migration and invasion, inhibited apoptosis, up-regulated expression of *Bcl-2*, and facilitated loss of *p27* in CHO (Botelho et al., 2009a,b, 2010, 2011a,b, 2013) – processes that are hallmarks of tumorigenesis and cancer cell survival (Hanahan and Weinberg, 2000). If similar phenomena also occur in human urogenital schistosomiasis, we speculate that they contribute to the abnormal proliferation and accumulation of genetic changes that occur in schistosomiasis-associated carcinogenesis [**Figure 2**; Mostafa et al., 1999; International Agency for Research on Cancer (IARC), 2012].

Opisthorchiasis is associated with elevation of bile acids, including deoxycholic acid (Vale et al., 2013) which are potent tumor promoters in cholangiocarcinogenesis (Sirica, 2005). Bile acids are synthesized in the liver from cholesterol, and the majorities are conjugated with either glycine or taurine (Haswell-Elkins et al., 1994; Ohshima et al., 1994; Akaike et al., 2003; Pinlaor et al., 2003; Katsuma et al., 2005; Sirica, 2005; Yongvanit et al., 2012). Inflammation-related carcinogenesis has also been associated to oxidative and nitrative DNA damage as 8-oxo-7,8-hydro-2′-deoxiguanine (8-oxodG) and 8-nitroguanine (8-NG; Yongvanit et al., 2012). Increased levels of nitrate and nitrite, which reflect endogenous generation of NO, occur during *O. viverrini* infection in humans (Haswell-Elkins et al., 1994) and rodents (Ohshima et al., 1994). Oxysterols, which are oxidation products of cholesterol generated by enzymatic (P450) or non-enzymatic processes (Jaworski et al., 2001; Jusakul et al., 2011), can be mutagenic or genotoxic, and to possess pro-oxidative and pro-inflammation properties that promote carcinogenesis. Investigation of binding domains in human genes has demonstrated an association between different types of oxysterols and the development and progression of cancer of the colon, lung, breast and bile ducts (Jaworski et al., 2001). Bile acids constitute a large family of steroids carrying a carboxyl group in the side chain. Bile alcohols have similar products in bile acid biosynthesis or as end products. We found these compounds in extracts of *O. viverrini* (**Figure 2** compound 18) but conjugated at different positions, free bile acids re-conjugated in some species like aldehydes (**Figure 2** compound 12) or as sulfates (not shown). The effects of these individual species can be anticipated to be structure-dependent, and metabolic conversions will result in a complex mixture of biologically active and inactive forms (Vale et al., 2013).

INFECTION WITH BLOOD FLUKES AND LIVER FLUKES AS THE RISK FACTOR – BUT HOW MIGHT CANCER ARISE?

Current understanding of how infections with these flukes lead to cancers has been reviewed recently (Sithithaworn et al., 2012; Sripa et al., 2012; Honeycutt et al., 2014). In brief, in regions of high prevalence of opisthorchiasis, the risk factors for bile duct cancers are chronic inflammation and concomitant chronic injury of the biliary epithelium as the consequence of persistent parasitism by these fish-borne pathogens (Sripa et al., 2009, 2012; Blechacz et al., 2011; Johnson et al., 2012; O'Hara et al., 2013; Razumilava and Gores, 2013). The risk of SSC of the bladder during urogenital schistosomiasis appears to be promoted by concurrent risk factors associated with bladder cancer where infection with *S. haematobium* is less common or in non-endemic regions including exposure to toxins such as dyes from industrial and agricultural sources, and from tobacco smoke (see Honeycutt et al., 2014). Thus there are likely to be multiple factors including a diet rich in nitrosamines, spillover effects from local and systemic chronic inflammation (reactive oxygen species, reactive nitrogen species) directed against the worms, the secretion of mitogens and other mediators by the parasite (Satarug et al., 1998; Sripa et al., 2012), and interactions or changes in the biliary, GI tract and urinary tract microbiota, including co-infection by other potentially oncogenic biological species (Plieskatt et al., 2013).

To this list, we now include another potential mechanism: lesions in chromosomes and production of depurinating estrogen-DNA adducts leading to parasite metabolite-promoted host cell DNA damage, due to parasite-derived, reactive oxysterol and/or catechol estrogen derivatives. These processes contribute to urogenital schistosomiasis associated SCC during chronic urogenital schistosomiasis, and to CCA during chronic opisthorchiasis (**Figure 2**). Overall, the structures that we have identified in *S. haematobium* and *O. viverrini* (Botelho et al., 2013; Vale et al., 2013) suggest that carcinogenesis-related steroids may be released in carcinogenic quantities by these flukes. Notably, a relation between putative oxysterol or bile acid metabolites from *O. viverrini* and bile duct cancer has long been hypothesized (Changbumrung et al., 1990).

CONCLUDING COMMENTS

Infection with helminth parasites remains a persistent public health problem in developing countries. Three of these pathogens, *C. sinensis*, *O. viverrini*, and *S. haematobium*, are of particular concern due to their classification by the IARC as Group 1 carcinogens. Infection with these worms is definitively associated with cancer. We have reported novel sterol-like metabolites and DNA-adducts in *S. haematobium*, in urine of persons with urogenital schistosomiasis, and in *O. viverrini*. Because these molecules are metabolized to active quinones that can modify DNA, helminth parasite associated catechol estrogens might induce tumor-like phenotypes in the epithelia of the bile ducts and bladder. Whereas the roles of these new metabolites in bile duct cancer and SSC of the bladder remain to be examined in depth, this clearly is worthy of deeper investigation. Future studies might profitably aim for isolation or chemical synthesis of these putative carcinogens and downstream investigation of interactions of the fluke estrogens and oxysterols with informative cells such as bladder urothelial cells (Botelho et al., 2013) and cholangiocytes (Grubman et al., 1994), and with oxysterol binding proteins and so forth. The interrelations of these carcinogens and the microbiota of the infected bladder and biliary system can also be predicted to be informative (Plieskatt et al., 2013). Moreover, given that other metabolites of *O. viverrini* are predicted to play a role in carcinogenesis of *O. viverrini* induced bile duct cancer, including liver fluke granulin (Smout et al., 2009), it will be informative also to compare and contrast action of liver fluke granulin and other fluke metabolites in these analyses, investigations that are now facilitated by the availability of genome sequences of these carcinogenic flukes (Wang et al., 2011; Young et al., 2012, 2014; Brindley and Hotez, 2013; Huang et al., 2013), genome sequences of CCAs (Chan-On et al., 2013), new rodent models (Fu et al., 2012), and functional genomic approaches developed for these parasites (Rinaldi et al., 2011, 2012). In addition to their carcinogenic effects, these flukes-associated sterol derivatives and DNA-adducts could be exploited as diagnostic and prognostic biomarkers, indeed 8-oxo dG in urine associates with opisthorchiasis-induced CCA (Thanan et al., 2008), and as targets for novel intervention strategies against these neglected tropical disease-associated cancers.

ACKNOWLEDGMENTS

José M. Correia da Costa, Maria J. Gouveia, Mónica C. Botelho, Lúcio L. Santos, and Júlio H. Santos thank FCT for Pest- OE/AGR/UI0211/2011 and Strategic Project UI211-2011-2013, Clínica Sagrada Esperança and Hospital Américo Boavida, Luanda, Angola. Nuno Vale thanks to Fundação para a Ciência e Tecnologia (FCT, Portugal) and FEDER (European Union) for funding through project grants CONCREEQ/275/QUI and PEst-C/QUI/UI0081/2011. Nuno Vale also thanks FCT for Post-Doc grant SFRH/BPD/48345/2008. The research findings reviewed here were supported by award R01CA155297 (Paul J. Brindley, Gabriel Rinaldi, Banchob Sripa) from the National Cancer Institute, NIH and P50 P50AI098639 (Banchob Sripa, Paul J. Brindley) from the National Institute of Allergy and Infectious Diseases, NIH. The contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 08 August 2014; paper pending published: 04 November 2014; accepted: 04 December 2014; published online: 23 December 2014.

*Citation: Correia da Costa JM, Vale N, Gouveia MJ, Botelho MC, Sripa B, Santos LL, Santos JH, Rinaldi G and Brindley PJ (2014) Schistosome and liver fluke derived catechol-estrogens and helminth associated cancers. Front. Genet. **5**:444. doi: 10.3389/fgene.2014.00444*

This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Genetics.

frontiers in
GENETICS

REVIEW ARTICLE
published: 10 September 2014
doi: 10.3389/fgene.2014.00317

Epigenetic control of gene function in schistosomes: a source of therapeutic targets?

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The discovery of the epigenetic regulation of gene expression has revolutionized both our understanding of how genomes function and approaches to the therapy of numerous pathologies. Schistosomes are metazoan parasites and as such utilize most, if not all the epigenetic mechanisms in play in their vertebrate hosts: histone variants, histone tail modifications, non-coding RNA and, perhaps, DNA methylation. Moreover, we are acquiring an increasing understanding of the ways in which these mechanisms come into play during the complex schistosome developmental program. In turn, interest in the actors involved in epigenetic mechanisms, particularly the enzymes that carry out epigenetic modifications of histones or nucleic acid, as therapeutic targets has been stimulated by the finding that their inhibitors exert profound effects, not only on survival, but also on the reproductive function of *Schistosoma mansoni*. Here, we review our current knowledge, and what we can infer, about the role of epigenetic mechanisms in schistosome development, differentiation and survival. We will consider which epigenetic actors can be targeted for drug discovery and what strategies can be employed to develop potent, selective inhibitors as drugs to cure schistosomiasis.

Keywords: epigenetics, schistosome, drug discovery, histone modifying enzymes, DNA methylation, microRNAs

INTRODUCTION

Schistosomiasis is caused by flatworm parasites of the genus *Schistosoma*, five species of which infect humans in 74 tropical and sub-tropical countries. It is estimated that more than 230 million people are infected, of which 90% are in sub-Saharan Africa (Colley et al., 2014 for review). Nearly 30 years after its introduction the treatment and control of schistosomiasis relies almost exclusively on praziquantel, the only drug effective against all schistosome species infecting humans. Its use has been and remains an unquestionable success story; mass treatments of school-age children in sub-Saharan Africa under the Schistosomiasis Control Initiative (Fenwick et al., 2009) hold the promise of a marked continent-wide reduction in disease morbidity and mortality. Nevertheless, the massive use of this drug may well-lead to the selection of resistant/tolerant parasite strains. Episodes of drug tolerance have been reported (Doenhoff et al., 2008; Melman et al., 2009) and can be induced readily in the laboratory (Fallon and Doenhoff, 1994). In addition, changes to the local genetic polymorphism of parasites following treatment of the population have been detected (Norton et al., 2010; Coeli et al., 2013), suggesting an effect of drug selection pressure. Although the precise mechanism of action of praziquantel in killing schistosomes is unknown, its initial effects include the rapid influx of Ca^{2+} ions and calcium-dependent muscle contraction and paralysis (Day et al., 1992) and this may be mediated via its interaction with a calcium channel beta subunit (Kohn et al., 2001). However, resistance to praziquantel may be mediated by increased expression of the P-glycoprotein efflux pump, which is often involved in drug resistance mechanisms, following exposure to praziquantel (Messerli et al., 2009). Whether or not such reports are the harbingers of the development of resistance by schistosomes toward praziquantel, reliance on a single drug is patently untenable in the medium to long term.

Most of the current efforts to identify new drug leads for schistosomiasis and other neglected parasitic diseases rely on the screening of random compound libraries directly on the parasite maintained in culture (phenotypic screening). The recent publication of the genome sequences of a variety of parasites including the three main species of schistosomes that infect humans (Berriman et al., 2009; Zhou et al., 2009; Young et al., 2012) now means that approaches targeting specific gene products or pathways can be envisaged. These can include enzymes with activities specific to the parasite, or at least not found in the human host (e.g., Sayed et al., 2008), metabolic bottlenecks, or molecules that are targeted in other pathologies. For these a wide knowledge base and extensive libraries of inhibitors may already exist that can be exploited as starting points for the development of parasite-selective compounds. This type of approach also has the advantage that the molecular mechanism of action of a given compound, which is required for any new drug, is much easier to determine than with the random screening approach. However, both strategies are still used and both have proved fruitful sources

of new drugs (Swinney and Anthony, 2011) although a more recent analysis of discovery of first-in-class drugs suggests a growing predominance of target-based approaches (Eder et al., 2014). Drug discovery is not a zero-sum game, but more a Nash equilibrium (Nash, 1950; Holt and Roth, 2004) in which the coexistence of strategies is not only possible but can also be highly productive.

Schistosomes are digenean parasites that successively infect freshwater snails (the intermediate host) and the vertebrate definitive host. They reproduce both asexually (within the snail host) and sexually (vertebrate host) and their life-cycle includes four distinct morphological forms and separate sexes at the adult worm stage (Colley et al., 2014). The complexity of schistosome development and differentiation implies a tight control of gene transcription at all stages of the life-cycle and that epigenetic mechanisms are likely to play a crucial role in these processes, suggesting that they are viable drug targets. In other pathologies, but most intensively in cancer, the targeting of epigenetic processes is increasingly exploited. Indeed, two histone deacetylase (HDAC) inhibitors have already been approved for use and a number of other candidate drugs are undergoing clinical trials (Arrowsmith et al., 2012). Moreover, large libraries of compounds that affect epigenetic actors are available for testing against parasites. Here, we will consider which epigenetic mechanisms can be targeted in schistosomes and what methodologies can be used to develop parasite-selective drug leads.

EPIGENETIC MECHANISMS AS DRUG TARGETS

The term "epigenetics" envelops a variety of heritable changes in gene expression that are linked to structural modifications of the chromatin, without changes to the DNA sequence. These include DNA methylation, reversible post-translational modifications of histones, histone variants, chromatin remodeling factors and non-coding RNAs. Viewed as potential targets, the most readily "druggable" are the enzymes that carry out DNA methylation and histone modifications, and increasingly, micro-RNAs (miRNAs) among the non-coding RNA categories (**Figure 1**).

The investigation of the role of epigenetic mechanisms in the control of gene transcription in schistosomes, and hence in biological processes like development and reproduction, is in its early stages. Nevertheless, the knowledge so far acquired, or inferred from the nature of schistosomes as invertebrate metazoan organisms and from a detailed analysis of the epigenetic actors encoded in their genomes, can be exploited to develop novel therapeutic strategies. Moreover, insights into schistosome epigenetic mechanisms has been gained from studies aimed at developing such strategies, including for example the characterization of the actions of inhibitors of histone modifying enzymes (HMEs), or from transcript knockdown studies. Here we will review the current state of knowledge of the epigenetic apparatus in schistosomes, including the still disputed significance of DNA methylation, the miRNA repertoire, the histone modifying enzyme complement and the potential for the development of novel drug treatments targeting these elements.

DNA METHYLATION

DNA methylation encompasses the methylation or hydroxymethylation of cytosine residues, mainly, but not exclusively within CpG dinucleotides (Baubec and Schübeler, 2014) and is an important epigenetic mark associated with gene repression. In vertebrates three DNA methyltransferases (Dnmts) establish (Dnmt3a and Dnmt3b) and maintain (Dnmt1) DNA methylation marks. A further member of this family, Dnmt2 is primarily a tRNA methyltransferase with only weak DNA methyltransferase activity (Schaefer and Lyko, 2010). Disruption of DNA methylation patterns is present in a variety of diseases, particularly in cancer in which many oncogenic pathways lead to Dnmt1 overexpression, an overall DNA hypomethylation concomitant with hypermethylation of tumor suppressor genes at CpG islands in the promoter regions. Agents that provoke DNA demethylation, such as 5-azacytidine and 5-aza-2′-deoxycytidine (decitabine) have been approved for use in myelodysplastic syndrome (Yoo and Jones, 2006), but their mode of action is complex. It involves conversion to a triphosphate metabolite, incorporation into DNA provoking a DNA damage response and covalent trapping of Dnmt isoforms, followed by proteolysis of the Dnmts, demethylation and the reactivation of the hypermethylated genes (Streseman and Lyko, 2008). The cytotoxicity of the 5-aza nucleosides and the lack of a direct inhibitory effect on Dnmts have led to the search for leads for new drug development. One example is laccaic acid A, a recently developed direct DNA-competitive inhibitor of Dnmt1 (Fagan et al., 2013).

DNA METHYLATION IN SCHISTOSOMES

The presence of functional DNA methylation marks in schistosome genomes is controversial. Early work in which Southern blot analysis was carried out for selected genes after digestion with methylcytosine tolerant or sensitive restriction enzyme isoschizomers (*Hpa*II and *Msp*I) showed no differences in the restriction profiles for adult male or female *S. mansoni* DNA (Fantappié et al., 2001). Moreover, the methylcytosine-dependent restriction endonuclease McrBC failed to digest *S. mansoni* DNA. However, a more recent study (Geyer et al., 2011) in which a variety of more sensitive methods including GC-MS, anti-methylcytosine antibodies and targeted bisulfite sequencing were used concluded that cytosine methylation was indeed present and a hypermethylated repetitive intron within a forkhead gene was characterized. The only DNA methyltransferase encoded in the schistosome genome is Dnmt2. The methyltransferases usually associated with DNA methylation; Dnmt1 and Dnmt3 orthologs are both absent. Dnmt 2 has only weak DNA methyltransferase activity but has robust methyltransferase activity toward $tRNA^{Asp}$ and other tRNAs (Goll et al., 2006). The diverse group of animal species (including *S. mansoni* and *Drosophila melanogaster*) that express only Dnmt2 have very low DNA methylation levels (Kraus and Reuter, 2011). However, Dnmt2 does retain some cytosine methyltransferase activity (Hermann et al., 2003) and Geyer et al. (2011) showed that siRNA knockdown of SmDnmt2 transcripts reduced overall methylcytosine levels in the schistosome genome. These authors have further suggested that cytosine methylation is conserved throughout the phylum Platyhelminthes (Geyer et al., 2013). Against this, a comprehensive study (Raddatz et al., 2013) using whole-genome bisulfite sequencing showed that the *S. mansoni* genome lacked a detectable DNA methylation pattern, even at the "hypermethylated" locus identified by Geyer et al. (2011).

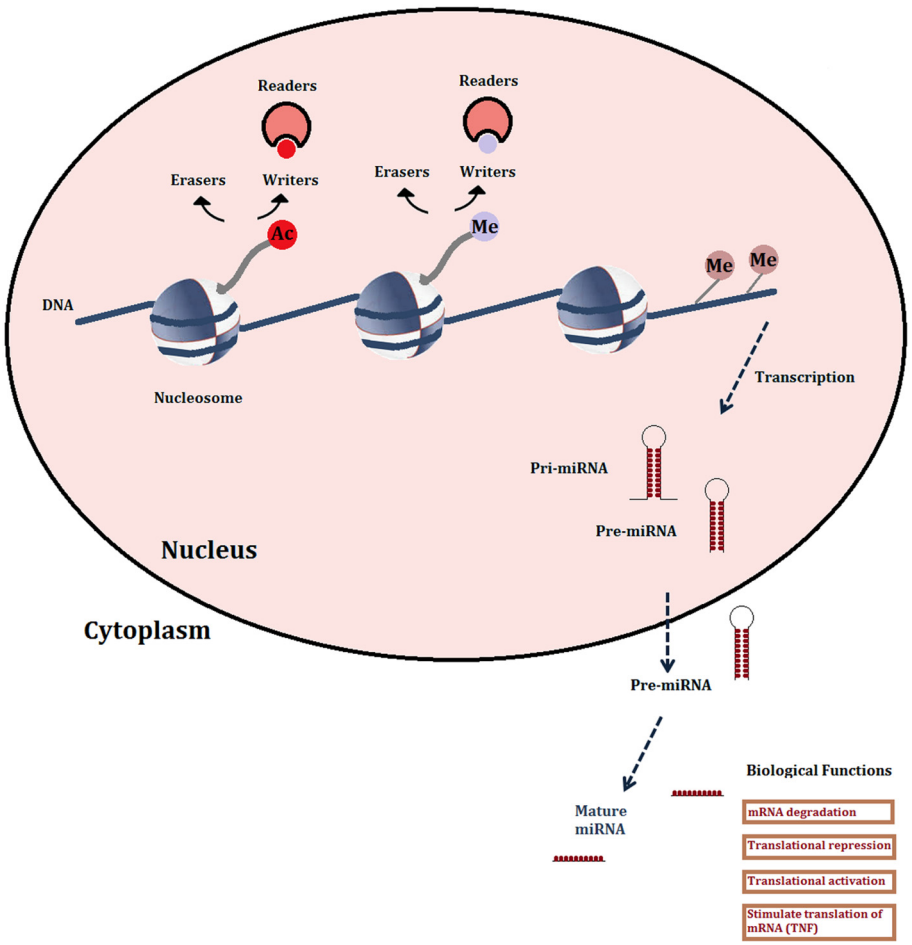

FIGURE 1 | Schematic representation of the major druggable epigenetic mechanisms. Histone modifications shown are limited to acetylation and methylation since the enzymes (writers and erasers) and recognition domains (readers: bromodomains) are the most studied for drug development. Also shown are DNA methylation and microRNAs.

Some clusters of incompletely converted cytosines were detected outside this region, but were consistent with bisulfite deamination artifacts (Warnecke et al., 2002). However, although these results strongly suggested that the *S. mansoni* genome is in fact unmethylated, the criticism has been leveled that the life-cycle stage analyzed, adult male worms, has the lowest level of DNA methylation measured using an ELISA method (Geyer et al., 2013). Notwithstanding this controversy, which will only be resolved by genome-wide bisulfite sequencing of other life-cycle stages, Dnmt inhibitors were found to strongly affect adult worms, particularly in terms of the morphology of the ovaries and *in vitro* egg-laying (Geyer et al., 2011). Whether or not this is due to the inhibition of DNA or tRNA methylation, it does suggest that Dnmt inhibitors such as 5-azacytidine may provide the basis for developing precursors of novel anti-schistosome drugs.

MICRO-RNAs

Non-coding (nc) RNAs include many different classes of transcripts that do not code for proteins, but have various regulatory roles in transcription, stability or translation of protein-coding genes. Of these, miRNAs are the best characterized in terms of their functional roles and pathological implications, as well as therapeutic strategies targeting them (Ling et al., 2013). They are generated from long, capped and polyadenylated transcripts that are processed by a nuclear complex containing RNase III (Drosha: canonical pathway) or by the mRNA splicing machinery (non-canonical pathway) (Li and Rana, 2014 for review) into 60–100 nucleotide precursors that are then transported into the cytoplasm where they are processed by the RNase Dicer into mature, double stranded miRNAs (**Figure 1**). Classically, miRNAs regulate transcript levels through binding to the 3′UTR regions of their target mRNAs, usually resulting in translational inhibition or mRNA destruction. However, it is now clear that miRNAs may have other mechanisms of action, for instance increasing translation via the recruitment of protein complexes to the mRNA or by binding proteins that block translation (Elring et al., 2010). Different miRNAs have been shown to have either tumor-suppressive (e.g., miR-15a-mIR-16-1 cluster) or oncogenic (mIR-21, mIR-17-mIR-92 cluster, mIR-155) properties.

Indeed, miRNAs can drive cancer: mIR 155 overexpression on its own provokes lymphoblastic leukemia or lymphoma in transgenic mice (Costinean et al., 2006). In cancer therapy, the upregulation of tumor-suppressive miRNAs has the advantage of simultaneously affecting a number of coding or non-coding genes that are targeted by the miRNA and that may be involved in the same or interacting pathways. A disadvantage is that a given miRNA may have different or even opposite effects in different cell types, depending on the expression patterns of its target genes. However, such considerations would be less of an obstacle in the therapy of parasitic diseases where it can be assumed that any disruption, positive or negative, of miRNA effects would be potentially deleterious to the parasite. Most current therapeutic strategies targeting miRNA in cancer are aimed at downregulating or blocking the function of oncogenic miRNA. One example consists in the use of antisense oligonucleotides, particularly those containing locked nucleic acids (LNA anti-mIRs) which are bicyclic RNA analogs in a locked configuration. One such compound, an anti-viral, miravirsen, is in clinical trials for the treatment of hepatitis C viral infection (Janssen et al., 2013; Lieberman and Sarnow, 2013).

SCHISTOSOME miRNAs

A survey of the available *S. mansoni* EST sequences (Oliveira et al., 2011) concluded that 10.3% (21,107 sequences) match the genome but have no protein coding potential and are therefore possible ncRNAs. This in turn suggests that the parasite may use a range of ncRNAs in transcriptional and translational regulation. Moreover, the presence of proteins involved in miRNA processing (Drosha, Dicer, and Argonaute) (reviewed in Oliveira et al., 2011) supports a role for miRNA regulation of development and differentiation of schistosomes and explains the effectiveness of RNAi and siRNA knockdown of transcription in the parasite (Boyle et al., 2003). miRNAs were first identified in *S. japonicum* (Xue et al., 2008; Huang et al., 2009) in two separate studies that demonstrated the existence of a limited number of miRNA that are conserved in other organisms including humans and several hundred novel miRNAs. In the second study all the novel miRNAs (172) were identified by an inferred RNA hairpin and many were differentially expressed during the life-cycle (Huang et al., 2009). Deep sequencing studies (Hao et al., 2010; Wang et al., 2010) further showed the presence of large numbers of siRNAs derived from transposable elements, but also identified 38 novel *S. japonicum* miRNAs. In *S. mansoni*, the sequencing of a small-RNA cDNA library yielded 211 novel miRNA candidates of which 11 were further verified by Northern blotting (Simoes et al., 2011). Therefore, although further work is necessary to validate the schistosome specific miRNAs and determine which of them are shared between schistosome species, it is clear that these miRNAs are potential therapeutic targets. It is of note that flatworms show a gradual loss of *conserved* miRNAs during evolution (Fromm et al., 2013), which is suggested to be due to morphological simplification. However, they have equally gained specific ncRNAs, including endogenous siRNAs, which are differentially expressed during development, notably during the sexual differentiation of female worms (Cai et al., 2011; Sun et al., 2014). A therapeutic strategy based on LNA anti-mIRs would have the advantage of targeting parasite-specific sequences and hence avoiding off-target effects, but it is not yet known whether individual miRNAs could be valid therapeutic targets. There are several additional challenges associated with such a strategy (Ling et al., 2013), the main ones being to ensure bioavailability to the parasite and oral delivery, which would require a significant effort to investigate appropriate chemical substitutions.

POST-TRANSLATIONAL MODIFICATIONS OF HISTONES

Histone post-translational modifications are currently under the most intensive study for drug development. The "writers" that add groups to histone N-terminal tails, "erasers" that remove them, or "readers" that recognize and bind them, are all potential therapeutic targets (**Figure 1**). The increasing variety of possible modifications includes phosphorylation, ubiquitinylation and sumoylation, but acetylation and methylation are the most abundant, most studied and their activity is mediated by the largest number of druggable proteins (Arrowsmith et al., 2012). Gene regulation is effected by combinations of these histone marks, leading to the "histone code" hypothesis (Strahl and Allis, 2000) whereby different chromatin states are defined by specific repertoires of marks.

Histone acetylation is a dynamic process regulated by histone acetyltransferases (HAT) that use acetyl-CoA as a co-factor and transfer an acetyl residue to the ε–amino group of lysines, particularly in the N-terminal tails of histones H3 and H4. The HDAC that remove this mark belong to four classes in mammals. Classes I, II, and IV have structurally-related catalytic domains and a Zn^{2+}-dependent catalytic mechanism (Gregoretti et al., 2004). The class III HDACs, or sirtuins, are phylogenetically unrelated and rely on NAD^{+} as a co-factor (Greiss and Gartner, 2009). Histone acetylation neutralizes the positive charge of the lysine, leading to a more relaxed structure permitting recruitment of the transcriptional machinery and in consequence is associated with transcriptional activation.

Histone methyl marks are written on lysine or arginine residues in histone tails by S-adenosylmethionine-dependent methyltransferases and erased by two classes of demethylase, the Jumonji family of demethylases that are 2-oxoglutarate-dependent, or the flavin-dependent lysine-specific demethylase 1 (KDM1/LSD1) and 2 (KDM2/LSD2). Unlike acetylation, a methyl group has no effect on the overall charge of the lysine or arginine residue that carries it and the effects of the mark are mediated by "reader" proteins that either compact the nucleosomes or form complexes with other regulatory proteins. Moreover, lysine residues can react with different reader domains depending on their position and degree (mono-, di, -or tri-) of methylation and can consequently integrate signal platforms determining activation or repression of transcription (Badeaux and Shi, 2013).

The current list of inhibitors of "Histone Modifying Enzymes" (HMEs) approved for use in humans or in clinical trials reflects the initial concentration in this field on the HDACs as therapeutic targets. Of 18 such compounds, 17 are HDAC inhibitors (including the two approved compounds, Vorinostat, and Romidepsin) and another is a sirtuin (Sirt) 1 inhibitor (Arrowsmith et al., 2012; West and Johnstone, 2014). Of the HDAC inhibitors in

clinical trials some inhibit class I and II enzymes indiscriminately, whilst others are more selective. Romidepsin preferentially inhibits the class I HDACs 1, 2, 3, and 8, whilst Vorinostat inhibits the class II HDAC6 and HDAC8 only poorly (Arrowsmith et al., 2012). Selectivity for a given HDAC or class may be of therapeutic importance as these enzymes have different targets. HDACs generally deacetylate both histones and other proteins. HDAC6 is not involved in epigenetic signaling at all but deacetylates tubulin and Hsp90 (Hubbert et al., 2002; Kovacs et al., 2005), while the only known HDAC8 substrate is SMC3, a component of the cohesin complex (Deardorff et al., 2012). HDAC inhibitors occupy the hydrophobic tunnel in the enzymes that accommodates the acetyllysine substrate and coordinate the zinc ion at the base of the tunnel, for example with a hydroxamate grouping as for Vorinostat. Selectivity can be based on differences between the make-up and architecture of the tunnel, or on the surface-accessible rim. However, within the cell HDACs are often part of multi-protein complexes that may alter substrate and inhibitor specificities compared to isolated recombinant proteins (Bantscheff et al., 2011) and this remark is likely even more pertinent in the case of other HMEs.

Of the seven mammalian NAD^+-dependent class III deacetylases, or sirtuins (Sirt) Sirts 1, 2, 3, 6, and 7 have been shown to possess deacetylase activity (Feldman et al., 2012), although Sirt6 is also a fatty acylase (Jiang et al., 2013). The other two sirtuins, 4 and 5 are both predominately mitochondrial. Sirt5 is a demalonylase and desuccinylase (Du et al., 2011) and has recently been shown to regulate a novel lysine modification, glutarylation (Tan et al., 2014). Sirt4 exhibits ADP-ribosyltransferase activity (Haigis et al., 2006). Sirtuin inhibitors have been developed against Sirts 1, 2, and 3 that couple the deacetylation reaction with the cleavage of NAD^+, liberating free nicotinamide. Inhibitors can bind either to the conserved NAD^+-binding C-pocket, like nicotinamide itself, or the acetyllysine peptide-binding cleft between the large and small domains of the enzyme (Yuan and Mamorstein, 2012), or both. In addition, since Sirt1 expression has been associated with increased lifespan and memory, allosteric activators such as resveratrol have been explored as therapeutic agents Hubbard and Sinclair, 2014). Only one sirtuin inhibitor, selisistat, is currently in clinical trials, however, for Huntington's disease (Arrowsmith et al., 2012).

HAT generally lack obvious druggable sites and few selective inhibitors are currently available. The HAT catalytic domains have a conserved organization around a central fold where the acetyl-CoA cofactor binds. The peptide substrate binding site in the only solved structure is shallow and solvent accessible, reducing its capacity to be targeted by drugs (Arrowsmith et al., 2012). Among the inhibitors so far described are natural substances that promiscuously bind a variety of targets (Piaz et al., 2011), or isothiazolone covalent modifiers (Ghizzoni et al., 2009). These latter include the more recently developed pyridoisothiazolones that effectively inhibit cancer cell proliferation (Furdas et al., 2011). However, a potent, selective inhibitor of the HAT EP300, C646, has been developed that binds at the cofactor pocket and has pro-apoptotic effects on prostate cancer cells (Bowers et al., 2010). This indicates that at least certain HATs are valid, standalone therapeutic targets, but effective screening may depend on the reconstitution of multi-protein complexes in which they are active in the cell and which may modulate their enzymatic activity (Arrowsmith et al., 2012).

The protein methyltransferases include both lysine (KMT) and arginine (PRMT) methyltransferases that are phylogenetically unrelated but share the requirement for S-adenosylmethionine as a cofactor and a cofactor binding site adjacent to the channel that binds the peptide substrate (Arrowsmith et al., 2012). Both these sites can be used to generate selective and potent inhibitors for both PRMTs and KMTs (Spannhoff et al., 2009; Dowden et al., 2010). However, in order to screen certain of the latter enzymes, the reconstitution of protein complexes is a prerequisite. The KMT component of the PRC2 transcription repression complex, EZH2, which methylates H3K27, is inactive on its own and minimally requires the presence of at least two members of the complex, EED and SUZ12 (Helin and Dhanak, 2013). High-throughput screening has been carried out on a complex additionally containing AEBP2 and RbAp48 proteins and has yielded highly selective inhibitors such as GSK126, which is a promising lead for the treatment of lymphoma (McCabe et al., 2012). Complex recomposition is not always necessary; the H3K79 methyltransferase DOT1L requires no partner proteins and a highly selective inhibitor, EPZ-5676, has been developed using a structure-guided strategy, with significant activity in a rat xenograft model of MLL-rearranged leukemia (Daigle et al., 2013).

Histone demethylases are under increasing scrutiny as drug targets (Hojfeldt et al., 2013). Two unrelated families of proteins exert demethylase activity, the LSD family and the JMJC domain-containing demethylases. In mammals the LSD (for Lysine-Specific Demethylase) family comprises only two members, but they share an amine oxidase-like domain with several metabolic enzymes in addition to a SWIRM (SW13, RSC8, and Moira) domain that is only found in some chromatin-associated enzymes. These enzymes use FAD as a cofactor that binds to one of two folded subdomains of the amine oxidase-like domain, the other binding the substrate. However, substrate specificity may be regulated through interactions of the SWIRM domain with protein partners (Metzger et al., 2005). The conservation of the amine oxidase-like domain means that some inhibitors of monoamine oxidases, such as tranylcypromine also inhibit LSD1 (Schenk et al., 2012), however, selectivity for the latter can be improved in derivatives. One such inhibitor, ORY-1001, has greatly improved selectivity for LSD1 and is entering clinical trials (Maes et al., 2013). Few selective or potent inhibitors of JMJC (Jumonji C) demethylases have yet been reported. In these enzymes (31 family members in humans) the JMJC domain is catalytic and the enzymatic mechanism involves two cofactors, Fe(II) and 2-oxoglutarate, which are bound to it. Members of this family can demethylate mono-, di- or tri-methylated lysines. Most reported inhibitors contain metal chelating groups (analogous to HDAC inhibitors, but the latter are poor JMJC inhibitors) that compete with the 2-oxoglutarate cofactor. One remarkably selective inhibitor of JMJD3, an H3K27me3-specific demethylase, has been developed using a structure-guided approach (Kruidenier et al., 2012). This inhibitor, GSK-J1, interacts with critical amino

acids involved in the binding of both the 2-oxoglutarate cofactor and the histone peptide substrate, and is competitive with the former but non-competitive with the latter. It is selective for both JMJD3 and Utx (a closely related JMJC demethylase active on the same substrate as JMJD3) but inactive against other members of the JMJC family. Moreover, GSK-J1 inhibits pro-inflammatory functions of human primary macrophages, indicating a possible therapeutic role. Moreover, a related JMJD3 inhibitor, GSK-J4, has recently been shown to have anti-tumorigenic activity against T-cell acute lymphoblastic leukemia (Ntziachristos et al., 2014).

Of the "readers" of epigenetic marks, bromodomain proteins, which read acetylated lysine residues, have so far attracted the most attention as drug targets. Bromodomains are composed of a characteristic antiparallel bundle of four α-helices that binds acetyllysine in a pocket at one extremity. They were first identified in the *Drosophila* gene brahma, hence the name, and in humans 61 bromodomains have been identified in 46 proteins, some containing more than one, and belong to eight distinct families (Hewings et al., 2012). Although the biological roles of most bromodomain proteins remain unknown, the connection of some of them with diseases such as cancer is becoming clearer (Arrowsmith et al., 2012; Filippakopoulos and Knapp, 2014). Small molecule inhibitors have been principally been developed against the BET bromodomain family. A triazolobenzidine (Nicodeme et al., 2010) was initially isolated as an inducer of ApoA1 expression and a derivative with enhanced activity in a reporter gene assay was only subsequently shown to interact with the bromodomains of BET family members BRD2, 3, and 4 using a chemoproteomic approach (Chung et al., 2011). Following an observation that thieodiazepines could bind to BRD4 a structure-based approach led to the development of a novel thieno-triazolo-1′4-diazepine named JQ1 (Filippakopoulos et al., 2011). This molecule binds to the BRD4 acetyllysine site and has reduced activity against other BRDs, but little or none toward other bromodomains. JQ1 induced a differentiation phenotype and growth arrest in a cell line derived from human squamous carcinoma. Since these founder studies, further selective inhibitors of BET bromodomains have been identified and clinical trials are ongoing, notably for JQ1.

SCHISTOSOME HISTONE MODIFICATIONS

An *in silico* analysis of the schistosome genome predictions and EST libraries (Anderson et al., 2012) showed that 21 of the 29 histone genes predicted in the genome are expressed in *S. mansoni*, the remainder being either unexpressed or having divergent sequences. Importantly, the N-terminal tails of the nucleosomal histones H3 and H4 are highly conserved, suggesting the functional conservation of the histone marks found in mammalian histones. The study of histone marks and their role in schistosome development and differentiation is still in its infancy, but studies involving inhibitors of HDACs and HATs have demonstrated the importance of histone acetylation and the interest of these enzymes as potential therapeutic targets. HDAC inhibitors including Trichostatin A (TSA) blocked the *in vitro* transformation of *S. mansoni* miracidia into primary sporocysts in a dose-dependent manner (Azzi et al., 2009) and this correlated with an increase in histone H4 acetylation. More recently, the same authors showed that differences in the levels of histone H3K9 acetylation on the promoters of genes encoding polymorphic mucins correlated with their differential expression in parasite strains compatible or incompatible with a given strain of the intermediate host, the freshwater snail *Biomphalaria glabrata*. TSA treatment ablated these strain-specific differences in expression (Perrin et al., 2013).

Histone acetyltransferase inhibition also has developmental consequences in schistosomes, particularly in egg maturation. The schistosome ortholog of the HAT GCN5 has been shown to acetylate H3 and H2A, and in particular H3K14 (de Moraes Maciel et al., 2008) and the CBP/P300 ortholog SmCBP1 primarily acetylates H4 (Bertin et al., 2006; Fantappié et al., 2008). Knockdown of either or both of these HATs in adult schistosomes has been shown to markedly reduce the transcription levels of the major eggshell protein p14 and to affect egg development. Moreover, these effects are reproduced by treating adult worm pairs with an HAT inhibitor, PU139 (Carneiro et al., 2014). After both inhibitor treatment and RNAi to knock down transcripts of the HATs, the phenotypic effects on egg laying and development were correlated with decreased acetylation of H3 and H4, increased methylation at H3K27, a marker of transcriptional repression, on the p14 proximal promoter.

The effects of both HDAC and HAT inhibitors on schistosomes suggest that histone acetylation may be a legitimate therapeutic target and this was further supported by a preliminary study showing that HDAC inhibitors like TSA and valproic acid could induce time and dose-dependent death of schistosomes (adult worms or schistosomula larvae) in culture (Dubois et al., 2009). Parasite death was associated with the induction of apoptosis in schistosomula shown by both TUNEL staining and the activation of the effector caspases 3/7. Once more, the molecular basis of these effects was evidenced by a global increase in histone H3 and H4 acetylation and significantly increased H4 acetylation at the proximal promoters of HDAC target genes, correlated with an increase in their transcription. More recently, similar effects have been observed using inhibitors of the class III HDAC, the sirtuins (Lancelot et al., 2013). Inhibitors of Sirtuins 1 and 2 such as salermide also induce apoptosis and death of schistosomula in culture. Moreover, salermide induces marked morphological alterations to the female worm genital apparatus, the arrest of egg-laying and the separation of worm pairs.

SCHISTOSOME HISTONE MODIFYING ENZYMES: WHICH ARE THE BEST TARGETS?

Schistosome HDACs and HATs are clearly potential drug targets, as are probably other HMEs, but there are several challenges that could potentially impede drug development:

- These enzymes are evolutionarily conserved, particularly their catalytic domains, and in order to avoid potential side effects, inhibitors that are selective for the schistosome enzyme have to be developed.

Table 1 | Identity and characteristics of *Schistosoma mansoni* histone modifying enzymes.

HME type	Class	Closest human ortholog	Size (aa)	Substrate specificity	Gene Id[a]
HDAC	I	HDAC1	517*		Smp_005210
	I	HDAC3	418*		Smp_093280
	I	HDAC8	440*		Smp_091990
	II	HDAC4	291		Smp_191310
	II	HDAC5	701		Smp_069380
	II	HDAC6	1132		Smp_138770
	III (Sirtuin)	Sirt1	568*	H1 – H3 – H4	Smp_138640
	III (Sirtuin)	Sirt2	337*	H4K16	Smp_084140
	III (Sirtuin)	Sirt5	305*		Smp_055090
	III (Sirtuin)	Sirt6	386*	H3K9 – H3K56	Smp_134630
	III (Sirtuin)	Sirt7	517*		Smp_024670
HAT	GNAT	GCN5 (KAT2A)	899*	H3K9 – H3K14 – H3K18 H2B	Smp_070190
	GNAT	HAT1 (KAT1)	435	H4K5 – H4K12	Smp_178700
	MYST	Tip60 (KAT5)	463	H2AK5 – H3K14 – H4K5 – H4K8 – H4K12 – H4K16	Smp_053140
	MYST	MYST1 (KAT8)	496	H4K16	Smp_194520
	MYST	MYST2 (KAT7)	400	H4K5 – H4K8 – H4K12 – H3	Smp_171700
	MYST	MYST3 (KAT6A)	971	H3K14	Smp_131320
	CBP/p300	CBP/SmCBP1 (KAT3A)	2093*	H2AK5 – H2BK15 – H3K14 – H3K18 – H4K5 – H4K8	Smp_105910
	CBP/p300	CBP/SmCBP2 (KAT3A)	1892	H2AK5 – H2BK15 – H3K14 – H3K18 – H4K5 – H4K8	Smp_127010
	TAFII250	TFIID subunit 1	2241	H3 – H4	Smp_166840
HMT	SET	EZH1	1026	H3K27	Smp_078900
	SET	MLL3 (KMT2C)	399	H3K4	Smp_070210
	SET	MLL3 (KMT2C)	1560	H3K4	Smp_138030
	SET	MLL1/4 (KMT2D)	3002	H3K4	Smp_144180
	SET	MLL5 (KMT2E)	751	H3K4	Smp_161010
	SET	C20orf11/MLL5/Ranbp9	1305		Smp_009980
	SET	NSD2/WHSC1	1746	H3K4 – H4K20	Smp_160700
	SET	NSD1/2 (KMT3B)	1343	H3K36 – H4K44	Smp_137060
	SET	SET8 (KMT5A)	409	H4K20	Smp_055310
	SET	SUV 39H2 (KMT1B)	586	H3K9	Smp_027300
	SET	SUV4-20H1 (KMT5C)	613	H4K20	Smp_062530
	SET	SETD2	1575	H3K36	Smp_133910
	SET	SETD1B	1720/1822	H3K4	Smp_140390
	SET	SETDB	918/1032		Smp_150850
	SET	SETMAR	250	H3K9	Smp_043580
	SET	SET/MYND4	782		Smp_000700
	SET	SET/MYND4	527		Smp_124950
	SET	SET/MYND5	423/429/433		Smp_121610
	DOT1	DOT1L (KMT4)		H3K79	Smp_165000
	PRMT	PRMT1	252/359/334	H4R3	Smp_029240
	PRMT	PRMT3	1564		Smp_127950
	PRMT	PRMT4/CARM1	737	H3R2 – H3R17 – H3R26	Smp_070340
	PRMT	PRMT5	630	H2A – H4	Smp_171150
	PRMT	PRMT7	755		Smp_025550
HDM	KDM1	LSD1A	1043		Smp_150560
	KDM1	LSD1A	916		Smp_160810
	KDM1	LSD1 (KDM1)	1073	H3K4 – H3K9	Smp_162940
	JmjC	JMJD1B (KDM3)	273	H3K9	Smp_161410
	JmjC	JMJD2C (KDM4C)	1136	H3K9 – H3K36	Smp_132170

(Continued)

Table 1 | Continued

HME type	Class	Closest human ortholog	Size (aa)	Substrate specificity	Gene Id[a]
	JmjC	JMJD4	809		Smp_147870
	JmjC	JMJD6	839		Smp_137240
	JmjC	JHDM1D (KDM7)	653	H3K36	Smp_127230
	JmjC	Jarid (KDM5)	2372	H3K4	Smp_156290
	JmjC	jarid (KDM5)	1639	H3K4	Smp_019170
	JmjC	UTX (KDM6A)	1137	H3K27	Smp_034000

**Validated by cDNA cloning.*

[a]Gene ID according to the genome annotation.

- If selective inhibitors can be developed, the targeted HME has to be essential to the parasite so that its inhibition is lethal. Many HMEs have overlapping specificities (**Table 1**) and the inhibition of one may be compensated by another. However, in the case of enzymes methylating (EZH) or demethylating (Utx) H3K27, only one isoform is present in schistosomes (**Table 1**), against two in humans, suggesting that these enzymes may represent particularly sensitive targets.

In consequence, for these particular therapeutic targets, an approach involving both target validation, notably by transcript knockdown (RNAi), and structural studies to determine specificities in the structure of the catalytic pocket, is essential.

The *S. mansoni* genome encodes 55 HMEs involved in protein acetylation/deacetylation or methylation/demethylation (**Table 1**) (Pierce et al., 2012). Some of these, including the class I HDACs (Oger et al., 2008) and the sirtuins (Lancelot et al., 2013) have been cloned and characterized and preliminary choices of targets can be made based on their degree of sequence conservation. In addition, the human orthologs of several of these enzymes are known to function only as part of a multiprotein complex as is the case for the H3K27 methyltransferase EZH2 mentioned previously (McCabe et al., 2012). High-throughput screening of the human enzyme has been done, using a five protein complex, but the resources devoted to carry out such a strategy for the development of anti-cancer therapies are not available in the case of neglected tropical diseases. Therefore, although the schistosome ortholog of EZH2 is a unique target, its screening will pose difficulties and it is therefore preferable to target an enzyme that is active on its own (like the H3K79 methyltransferase DOT1L for example Daigle et al., 2013). In addition, certain HMEs are very large proteins (**Table 1**) and although the production of truncated proteins containing the catalytic domain can be envisaged, this may affect both enzyme activity and the conformation of the catalytic pocket, limiting the relevance of screening or structural data.

These considerations reduce the choice of viable HME targets in schistosomes, but a large number remain. Two filters can be used to further limit the choice: the use of HME class inhibitors to determine whether enzyme families contain potential targets and transcript knockdown using RNAi to validate individual HMEs as stand-alone therapeutic targets. RNAi is still the only available means to achieve targeted knockdown of gene function in schistosomes, but its efficacy is transcript-dependent and phenotypes are not always observed (Stefanic et al., 2010).

DEVELOPMENT OF SELECTIVE INHIBITORS AS DRUGS: THE CHALLENGES

An illustration of the strategy that can be employed to designate therapeutic targets is provided by the *S. mansoni* class I histone deacetylase 8 (SmHDAC8). Of the class I HDACs, SmHDAC8 was initially designated as a potential target for two reasons. First, transcript expression levels of SmHDAC8 are higher than those of SmHDAC1 and SmHDAC3 throughout the life cycle, notably in adult female worms (Oger et al., 2008). It is notable that HDAC 8 transcript levels are generally much lower than those of HDAC1 and 3 in normal human cells, but are markedly upregulated in some cancer cell lines and tissues (Nakagawa et al., 2007). Second, the analysis of the primary sequence of SmHDAC8 showed that it is less well-conserved compared to its human ortholog than the other two class I enzymes. This is demonstrated by the sequence alignment and phylogenetic analysis shown in **Figure 2**. The alignment shows that the essential residues for HDAC activity are conserved, but that the catalytic domain sequence contains insertions and substitutions that might indicate a change in architecture of the catalytic pocket, notably the replacement of a methionine (M274) in the human HDAC8 by a histidine (H292) in SmHDAC8. The status of SmHDAC8 as a stand-alone therapeutic target was enhanced by the use of generic HDAC8 inhibitors (unpublished results) and particularly by transcript knockdown using RNA interference (Marek et al., 2013). The latter showed that treatment of schistosomula with double-stranded RNA, followed by their injection i.v. into mice and harvesting of surviving worms 35 days later, led to a reduced worm recovery compared to mice treated with dsRNA encoding green fluorescent protein as a control.

Whilst molecular modeling seemed to show that only the charge difference within the catalytic pocket provided by His292 differentiated SmHDAC8 from its human counterpart, structural analysis by X-ray crystallography demonstrated a further important difference (Marek et al., 2013). In the schistosome enzyme, amino acid substitutions surrounding the catalytic pocket allow a change in the configuration of the side chain of phenylalanine 151. The side chain of the equivalent residue in human HDAC8, Phe152, adopts an obligatory flipped-in conformation that contributes to the narrow, hydrophobic tunnel accommodating the substrate or inhibitors. In contrast, in SmHDAC8 this side chain is free to adopt a flipped-out configuration, allowing the pocket to accommodate more bulky substrates or inhibitors. This difference, together with the charge difference, allows the possibility of identifying selective inhibitors for the schistosome

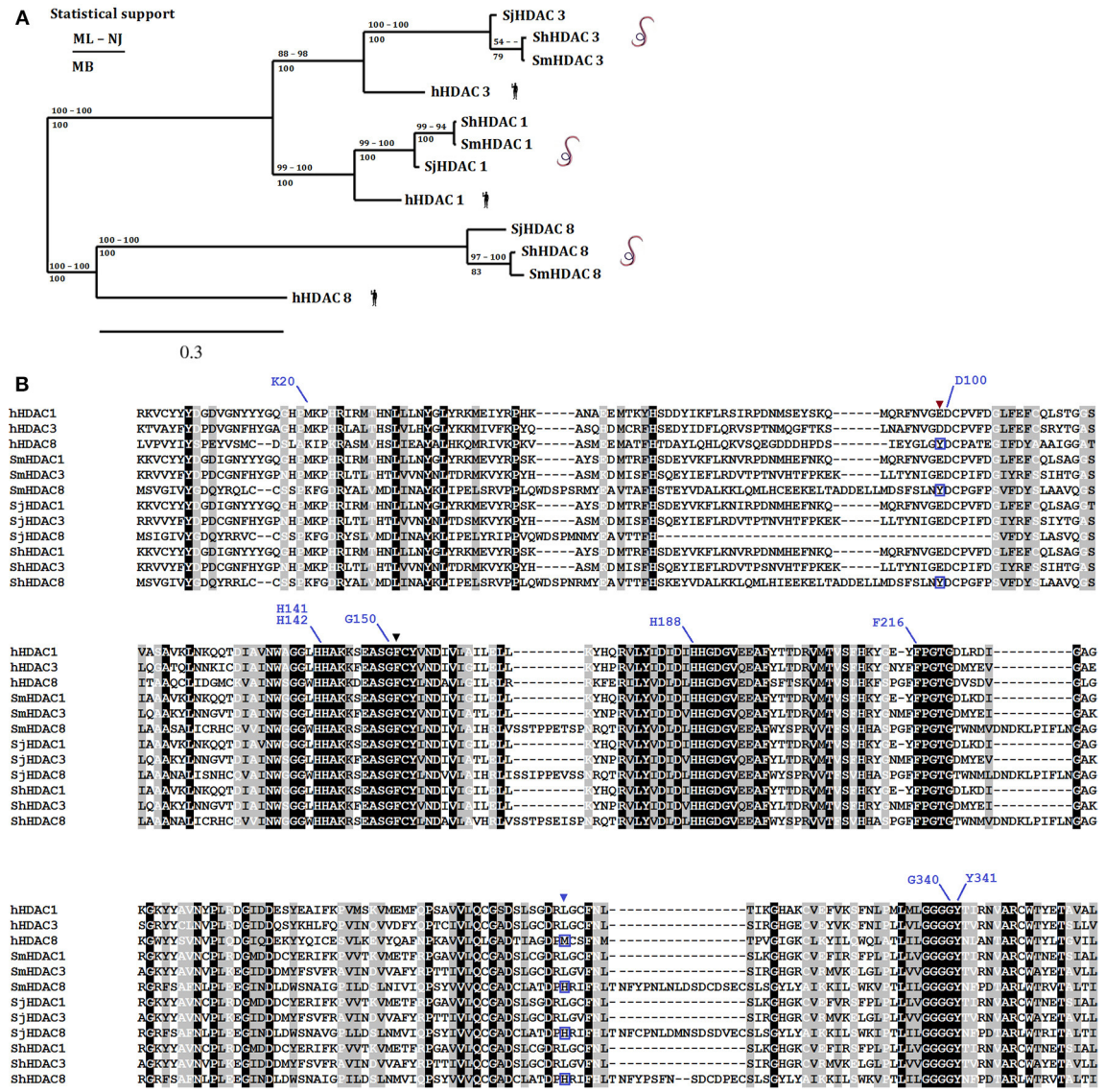

FIGURE 2 | Phylogenetic relationship amongst human and Schistosome class I HDACs. Panel **(A)** displays a phylogenetic tree [ML, consensus from maximum likelihood; NJ, neighbor joining; and MB, Mr Bayes methods) built using the amino acid sequences from the catalytic domains of class I HDACs (HDAC1, 3, and 8) present in *S. mansoni* (Sm), *S. japonicum* (Sj), and *S. haematobium* (Sh) and their human (h) orthologs. Panel **(B)** shows an amino acid sequence alignment constructed with MAFFT with the class I HDACs used above. Conserved (black background and white letters), conservative changes (gray background and black letters) and less conservative changes (gray background and white letters) in amino acid positions are highlighted. Black (F151) and brown (Y99) triangles show residues that despite being conserved present conformational differences between human and Schistosomes HDAC8 (Marek et al., 2013). The blue triangle shows the position of the replacement of a methionine (M274) in the human HDAC8 by a histidine (H292) in SmHDAC8 (boxed amino acids below blue triangle). Residues that interact with the SmHDAC8 specific inhibitor J1075 are highlighted with blue lines.

enzyme. Indeed, an *in silico* screen, based on the crystal structure of SmHDAC8 and involving the docking of a large number of potential inhibitors, led to the identification of an inhibitor, J1075 (Marek et al., 2013), which had greatly improved selectivity for SmHDAC8 compared to human class I and II HDACs. Moreover, this inhibitor caused dose and time-dependent death of schistosome larvae in culture via the induction of apoptosis. Optimization of this and other inhibitors identified by this strategy is ongoing, and further potential drug precursors have been identified (Stolfa et al., 2014). It is also notable that the structural specificities of the SmHDAC8 enzyme compared to the human ortholog are shared with HDAC8 in other flatworms, including other schistosome species (**Figure 2**), *Echinococcus* sp. and *Clonorchis sinensis* (Marek et al., 2013). Therefore, drugs effective against *S. mansoni* may well be applicable to these other species.

This example proves the concept that individual epigenetic enzymes can be valid therapeutic targets, and that, even though

these enzymes generally have conserved catalytic domains, sufficient differences in structure can exist to allow the development of selective inhibitors that are drug precursors. It remains to be seen, however, whether these inhibitors can be made sufficiently selective to preclude potentially harmful side effects and whether they can be developed into drugs useable in a single oral dose in humans.

CONCLUSIONS

Epigenetic processes provide a wealth of potential therapeutic targets for the development of novel therapies against schistosomiasis and other parasitic diseases. The most readily exploitable of these targets are the HMEs, as well as perhaps Dnmt2, which lend themselves to target-based drug discovery strategies, necessary to ensure the development of parasite-selective drugs. A structure-based strategy has been initiated for *S. mansoni* HDAC8, involving the solution of the 3D structure of the catalytic domain and *in silico* docking of potential inhibitors. However, such enzymes can also be screened directly using random compound libraries and high-throughput methodologies using enzyme inhibition as the read-out. Moreover, the existing extensive libraries of HME inhibitors can be used for phenotypic screening of compound libraries for lethal effects on the parasite itself. In all these cases, since the molecular mechanism of action of the drug precursor is known, time will be saved in the process of optimizing selectivity.

The remaining potential targets discussed above, notably the histone modification readers, like bromodomain proteins, and miRNAs, require validation as effective targets and pose greater challenges for drug development. In order to identify the schistosome bromodomain proteins, an exhaustive analysis of the genomic data has still to be done. However, since both bromodomain proteins and miRNAs are under intensive investigation, particularly for the development of anti-cancer therapies, methodologies will be developed that could be exploited and adapted for the treatment of parasitic diseases.

Work on all the potential targets discussed here can benefit from the increasing knowledge base and compound libraries accrued, notably in the development of anti-cancer therapies. This "piggy-backing" approach (Dissous and Grevelding, 2010) holds great promise and can in part mitigate the relative lack of investment in efforts to improve the control and treatment of schistosomiasis and the other neglected parasitic diseases.

ACKNOWLEDGMENTS

This work and the authors of this manuscript received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreements nos. 241865 (SEtTReND) and 602080 (A-ParaDDisE). The work of Julien Lancelot, Stéphanie Caby, Raymond J. Pierce, and Alejandro Cabezas-Cruz was also supported by institutional funds from the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSERM) and the Université de Lille 2. GO was also supported by contracts CNPq 573839/2008-5, 403049/20121, and 309312/2012-4), CAPES (070/13), and FAPEMIG (PPM-00439-10).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 June 2014; accepted: 24 August 2014; published online: 10 September 2014.

*Citation: Cabezas-Cruz A, Lancelot J, Caby S, Oliveira G and Pierce RJ (2014) Epigenetic control of gene function in schistosomes: a source of therapeutic targets? Front. Genet. **5**:317. doi: 10.3389/fgene.2014.00317*

This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Genetics.

frontiers in
GENETICS

MINI REVIEW ARTICLE
published: 08 September 2014
doi: 10.3389/fgene.2014.00301

Chromosomal differentiation of schistosomes: what is the message?

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As the only group of flukes with dioecism, schistosomes are unique organisms; they not only have intriguing biological and evolutionary aspects but also are responsible for major public health problems in the developing world. Schistosomiasis caused by this fluke affects approximately 210 million people in 76 countries. In order to facilitate the discovery of eradication methods for this disease, fundamental biological outcomes must be made available. Whole genome sequence data represent one such resource applicable to discovering eradication methods and measures. Herein, I describe three remarkable chromosomal changes and briefly discuss the differentiation of the Asian and African groups of this parasite taxon. Chromosomal data and evolutionary aspects will enable us to exploit genomic information for advancing schistosome studies.

Keywords: chiasma frequency, constitutive heterochromatin, telomere squence localization, geographical distribution, schistosomes

CHIASMA FREQUENCY

When I observed the meiotic cell division of *Schistosoma japonicum* for the first time, I was amazed at the differences in the shape of the chiasmatic formation of *S. mansoni* (see **Figures 1A,B**). **Figures 1A,B** highlight the differences in the number of chiasmata between the two human schistosome species, 20 for *S. mansoni* (A) and five for *S. japonicum* (B) *S. mansoni* has several chiasmata in each chromosomal arm, but there are only a few in the chromosomal arms of *S. japonicum*, though some terminal (end-to-end) associations were observed. In our previous study, the mean frequencies of chiasmata found within arms (FXi) were 15.3 for the *S. mansoni* Puerto Rican strain and, remarkably, only 3.0 for the *S. japonicum* Japanese strain (Hirai et al., 1996). This investigation revealed a clearly different situation in chiasmatic formation between these species. In addition to this difference, Asian schistosome species showed a regional cline of FXi rate of chiasma frequency; specifically, the values for *S. japonicum* Leyte, *S. japonicum* Mindanao, *S. japonicum* Luson, *S. japonicum* Anhui (China), *S. mekongi*, and *S. malayensis* were 3.6, 7.2, 7.5, 6.3, 8.6, and 9.0, respectively (Hirai et al., 2000).

What is the biological meaning hidden in these differences in chiasmata frequency? Chiasmata are chromosomal phenotypes of crossing-over (gene shuffling) between homologous chromosomes (John, 1990). Does the difference in the rate of crossing-over inform us of any significant relationships between the gene shuffling rate and evolutionary history in schistosome species? Because these differences in the FXi were significant, there most likely are biological meanings in the difference between *S. mansoni* and *S. japonicum* and among regional races of *S. japonicum*. A method to determine what differentiates species and races would be helpful, though a viable method is not yet available.

A sophisticated technique is not necessary to observe meiotic cell division, and only two dissection needles are required. I have used the following effective procedure, which is useful for observing chiasmata of schistosomes. It is possible to observe chromosomes of small organisms like schistosomes using this technique. Only the tiny testes and ovaries (each about one millimeter in length) of the adult schistosome worms with a body length of approximately 6–26 mm (**Figure 1C**) can be used to observe meiotic chromosomes. Briefly, the order of the procedure is as follows (**Figure 1D**): (a) dissect out the testes, (b) remove other tissues, (c) treat with a hypotonic solution (0.005% colchicine in 1% sodium citrate) on a culture glass slide for 30 min at room temperature, (d) transfer the testes to and pre-fixate with Fixative I (60% acetic acid:ethanol = 1:3) on a glass slide, (e) remove surplus solution and re-fixate with Fixative I, (g) tease the testes with two needles, (h) first, spread cells with drops of Fixative II (acetic acid:ethanol = 1:1), (i) second, spread cells with Fixative III (acetic acid only) after spreading out Fixative II, and (j) finally, desiccate at room temperature. For the details of this procedure, see Hirai and Hirai (2004). This technique is useful to make dry chromosome preparations from small organs, which are then available to clearly observe several stages of meiotic cell division. We were able to utilize silver nitrate (Ag) staining to detect the synaptonemal complex (SC; **Figures 1A,B**) and fluorescence *in situ* hybridization (FISH); thus, the chromosome preparations are readily adaptable for different techniques. The comparisons of gene order and genome structure between the Z and W chromosomes using the FISH technique are very useful (Hirai et al., 1989; Spotila et al., 1989; Criscione et al., 2009). When no dried chromosome preparations were available, I could not detect differences in chiasmata among schistosome species. In particular, the chiasmata frequencies of *S. mansoni* and *S. japonicum* were quite different from each other, and Asian species showed cline gradation of change in chiasmata frequency (Hirai et al., 2000).

Genetic crossing-over and chiasma formation are chromosomal actions to recombine maternal and paternal genetic elements

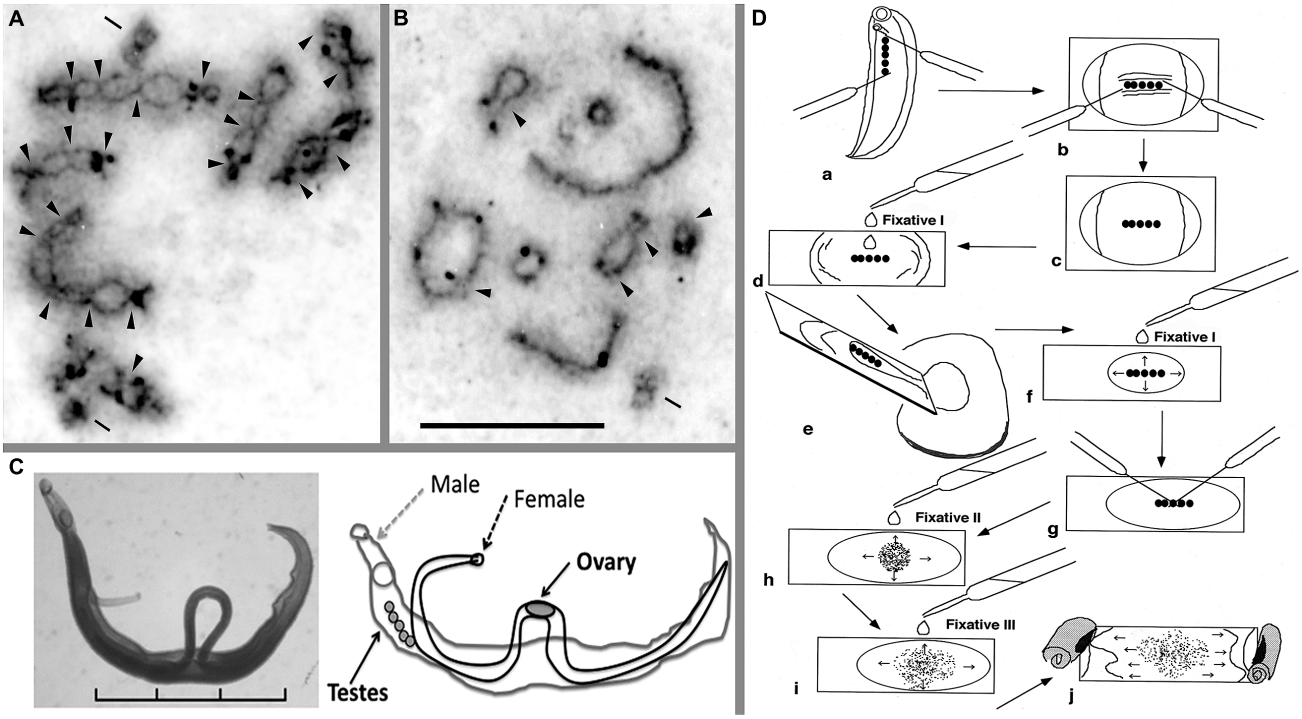

FIGURE 1 | Meiotic chromosomes, adult worms, and a technique for chromosome observation of schistosomes. Meiotic diakinesis stained by AG for *Schistosoma mansoni* **(A)** and *S. japonicum* **(B)**. AG stain shows the synaptonemal complex (SC) protein, which detects crossing over between two homologous chromosomes. **(C)** Pairing form of the adult female and male of *S. mansoni* and the schematic illustration. **(D)** A manual preparation to obtain meiotic chromosomes from adult schistosome worms. This schema shows an example of the process with a male worm. For details, see text. Arrowhead indicates chiasma region; bar shows centriole that was stained by AG. Scale of **(A,B)** is 10 μm. Scale in **(C)** is 3 mm. See also Hirai et al. (1996, 2000) and Hirai and Hirai (2004).

at the next generation. That is, the action shuffles gene order inherited from the ancestor of the lineage. The shuffle can be considered gene shuffling, which is useful to develop diversity in populations or lineages. Because crossovers and chiasmata are distributed non-randomly, it is the possibility for them to work as promotional or interference mechanisms at various levels. Differences in gene shuffling may produce the differentiation of several genetic traits.

CONSTITUTIVE HETEROCHROMATIN (C-BAND)

Mitotic chromosomes of schistosomes, which can be prepared using sporocyst stages infected in a snail host by another technique (see Hirai and Hirai, 2004), can be used with the staining method for constitutive heterochromatin (C-banding) to detect karyotypes, but not for G-banding that is available in higher order organisms like mammals. That is, G-bands are not consistently obtained as markers and are very unstable in schistosomes. Nonetheless, size, shape, and C-banding stains have therefore been used to identify chromosomes of schistosomes. I have used the TAM system (based on the non-random localization of the centromere; Imai, 1991) to classify C-banded karyotypes (see Hirai et al., 2000), which is a method to describe chromosome morphology using the existence mode of C-bands. As described in a previous paper, for example, the C-banded chromosome morphology (**Figure 2A**) of *S. haematobium* is designated as $1A^{e}$, $2A^{ec/}$, $3A^{e}$, $4A^{e}$, $5M^{/c}$, 6M, $7M^{/i}$, $ZA^{e/it}$, and $WA^{ec/ci}$ (Hirai et al., 2000).

Based on the representative C-band designation of *S. haematobium* chromosomes, five other species of schistosomes were also detected via karyotypes using the same nomenclature as in previous descriptions (see Figure 2 of Hirai et al., 2000). Comparing the changes in C-band patterns among six species revealed several meaningful patterns. **Figure 2B** shows this manifestation as a black-white gradation (analogy) and pattern (type of C-band). The similarity and dissimilarity of each chromosome allowed us to detect the gap and continuity of gradation patterns. Consequently, *S. haematobium* and *S. mansoni* showed higher similarity, and *S. japonicum*, *S. mekongi*, and *S. malayensis* showed similar patterns. The former and latter groups can thus be identified as African and Asian groups, respectively. In addition, *S. sinensium* appears as a mixed pattern and, therefore, is postulated to be a complex between Asian and African schistosomes.

Although chromosome paint analysis (Hirai et al., 2012) became a useful technique for identifying chromosomes of schistosomes (*S. mansoni*) in place of C-band analysis, C-banding remains valuable for investigation of chromosome evolution because the amount of heterochromatin and altered pathway of karyotypes (shape) can be ascertained only from C-band patterns. Because paint analysis is available for detecting translocation among non-homologous chromosomes, however, the

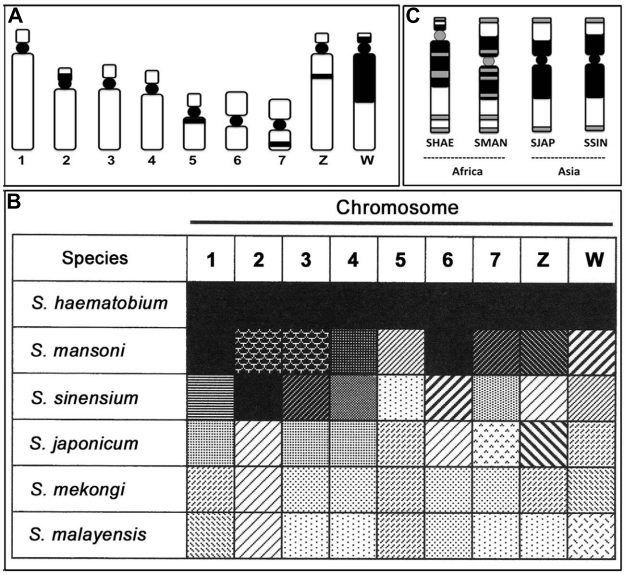

FIGURE 2 | Characteristics of constitutive heterochromatin (C-band) and telomere localizations of schistosome species. (A) A representative C-band pattern of seven autosomes and the Z and W sex chromosomes in *S. haematobium*. **(B)** Similarity and dissimilarity of C-band patterns among six schistosome species. The black and white gradation shows the grade of resemblance, and the pattern indicates similarity of the C-band and type in each chromosome of the six schistosomes. **(C)** Localization of telomere sequence in the W chromosome of *S. haematobium* (SHAE), *S. mansoni* (SMAN), *S. japonicum* (SJAP), and *S. sinensium* (SSIN). Black represents the C-band, and gray represents the positive site to the telomere sequence in **(A,C)**. See also Hirai et al., 2000.

combinational use of chromosome paint and C-band analyses is effective for chromosome analyses of schistosomes.

LOCALIZATION OF TELOMERE SEQUENCE

The telomere sequence motif of schistosomes is the same as that of humans (TTAGGG; Hirai and LoVerde, 1996); the location is thus detected with a probe $(CCCTAA)_7$ (Hirai et al., 2000). The localization pattern of the telomere sequence indicated specificity in the W chromosomes of four species (*S. haematobium*, *S. mansoni*, *S. japonicum*, and *S. sinensium*). Generally, tandem repeats of the telomere sequence are located at the very end of chromosomes. However, telomere sequence integration into some other regions has been observed in several species groups (e.g., Meyne et al., 1990; Go et al., 2000). Schistosome species display differences in the W chromosome between African and Asian groups. The African species showed telomere signals not only in the end regions but also in the large heterochromatin block and centromere of the W chromosome (multiple type), whereas the Asian species showed signals only in the end regions (simple type; **Figure 2C**). Accordingly, telomere sequence localization in the W chromosome also can distinguish the locality of schistosome species for African and Asian species based on the multiple and simple types of telomere localization. That is, the localization features of telomere sequences are also useful markers to determine the geographical distribution of schistosomes.

VIEW

The study of schistosome chromosomes was pioneered by Short (1983); their work formed the basis for developing new chromosomal techniques and studies for the species, where technical challenges previously existed in the preparation of chromosome smears. We have modified the techniques for use with molecular chromosome analyses (Hirai and Hirai, 2004). Eventually, such chromosomal techniques were able to support genome sequence analyses (Berriman et al., 2009). At that point, new findings were made in the following areas: localization of repeat and female-specific DNA, telomere sequences, localization of YAC and BAC clones, gene localization, chromosome paint analysis, and evolution of the W chromosome, among others (e.g., Hirai et al., 1989, 1993, 1996, 2012; Hirai and LoVerde, 1995, 1996). Additionally, localization of molecular markers has supported genetic analyses, for example, linkage mapping (Criscione et al., 2009), which will be informative in the next generation of research on functional genomics in schistosomes. During these investigations, a surprising finding was the dramatic difference in chiasma formation among schistosome species (Hirai et al., 1996, 2000). Although the influence of this differentiation on biological manifestation remains a mystery, recently established genetic analyses (Criscione et al., 2009; Tait, 2009) have the potential to decipher the meaning and role of differences in chiasmata. Investigations related to differentiation of chiasmata will rapidly progress with application of the new genetic techniques as well as the new generation of genome sequencing. The progress made with these techniques will help advance several areas of study, including linkage disequilibrium studies, sperm typing, genome-wide patterns of recombination, detection of hotspots of recombination, and the HapMap project.

As an example of such developments, using the assumption that deletion of DNA segments is more difficult than their addition, chromosomal differentiations can indicate the direction of the change from Asian to African species groups of schistosomes. That is, African schistosomes have an insertion of a telomere sequence in some parts of the heterochromatin block and in the centromere of the W chromosome, a trait that is not observed in Asian species (**Figure 2C**). Hypothetically, deletion of all such insertions is almost impossible, even if additional insertions are possible. A hypothetical pathway of change on chromosome 2 also showed the same direction as the telomere condition (Hirai et al., 2000). If we follow this pathway, comprehensive C-band patterns also show a direction from lighter to darker gradation (**Figure 2B**). The direction of these differentiations is consistent with molecular data as well. That is, comparisons of DNA sequences strongly support that the genus *Schistosoma* originated in Asia and then distributed to Africa, indicating that there is an Asian origin for *Schistosoma* (Le et al., 2000; Attwood et al., 2002; Agatsuma, 2003). If these inferences are correct, chiasma frequency also differs from a smaller to a larger number of chiasmata from Asia to Africa, respectively (**Figures 1A,B**). Chiasma is a biologically influential mechanism of gene shuffling between homologous chromosomes in the meiotic phase and is important to ensure the maintenance of diversity in a population. A lower chiasma frequency (Asian types) results in less gene shuffling than with a larger frequency (African type). This may work to increase

the breadth of biological differentiation between Asian and African schistosomes.

S. sinensium, described as a new schistosome species in China (Pao, 1959), is an interesting Asian species. The species seems to have derived from a common ancestor of both groups of *S. japonicum* and *S. mansoni* (Greer et al., 1989), a concept also supported by molecular analyses (Agatsuma et al., 2001). Chromosomes of *S. sinensium* also showed a mixed suite of C-band patterns (**Figure 2B**). Specifically, chromosomes 2, 3, and 4 are similar to those of *S. haematobium* and *S. mansoni* (African type), whereas chromosomes 1, 5, 7, Z, and W are similar to those of *S. japonicum*, *S. mekongi*, and *S. malayaensis* (Asian type). In addition, telomere localization in the C-band block of the W chromosome is the same as that of *S. japonicum* (**Figure 2C**). That is, this species shows a complex manifestation between Asian and African species groups not only at the morphological and molecular levels but also at the chromosomal level.

Genome sequencing in the representative three species of schistosome flukes (*S. mansoni*, *S. japonicum*, and *S. haematobium*) has opened routes to new insights and developments in biology and control of human schistosomiasis (Berriman et al., 2009; The *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium, 2009; Young et al., 2012). These projects clarified many genes that can be used in the eradication of disease and development of drugs to control the disease. In addition, differences in the frequency of repetitive sequence were detected between the three species. The role of chiasma as an important factor in shuffling the gene structure in a genome is diminished by the existence of constitutive heterochromatin (C-band). Heterochromatin often shows species specificity because it is prone to change rapidly via the repetitive sequence, and it influences chiasma formation in many organisms (Verma, 1988). In the genome projects, the amount of repetitive elements was estimated as follows: 40% for *S. mansoni*, 40.1% for *S. japonicum*, and 43% for *S. haematobium*. We are not yet able to find direct relationships among gene characteristics, chiasma formation, amount of repetitive sequences, C-band variation, telomere sequence localization, geographical distribution, speciation, disease states, and so forth. If we apply novel generation analyses as mentioned above, however, such comprehensive biological information would undoubtedly aid in the control and even eradication of these neglected parasite diseases. For instance, determining meiotic recombination hotspots may also provide critical information for genetic differentiation, evolution, and disease control. Such sophisticated analyses are required for further studies with schistosome chromosomes and should be possible given that these approaches have already been expanded with human chromosome analyses, which are more difficult than in model organisms like mice or yeast (e.g., Egel and Lankenau, 2008).

CONCLUSION

Until now, chiasma formation and crossing-over – genetic recombination – have not been investigated in schistosomes. They could not be previously analyzed in taxa because of the lack of methodology and technology. However, as mentioned above, such required techniques and methods are ready to be used presently. Comprehensive studies related to genetic and genomic analyses should be developed to precisely assess the features of biological interest and medically important organisms to eradicate menaces of schistosomes.

ACKNOWLEDGMENTS

I thank Yuriko Hirai for the great technical assistance with the molecular chromosome analyses and Phil LoVerde for giving me the opportunity to pursue the molecular cytogenetic approach with schistosomes. I am also grateful to many colleagues with whom I have had wonderful collaborations in schistosome studies.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 July 2014; accepted: 12 August 2014; published online: 08 September 2014. Citation: Hirai H (2014) Chromosomal differentiation of schistosomes: what is the message? Front. Genet. ***5****:301. doi: 10.3389/fgene.2014.00301*

This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Genetics.

frontiers in GENETICS

REVIEW ARTICLE
published: 05 August 2014
doi: 10.3389/fgene.2014.00262

Deciphering the glycogenome of schistosomes

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Schistosoma mansoni and other *Schistosoma* sp. are multicellular parasitic helminths (worms) that infect humans and mammals worldwide. Infection by these parasites, which results in developmental maturation and sexual differentiation of the worms over a period of 5–6 weeks, induces antibodies to glycan antigens expressed in surface and secreted glycoproteins and glycolipids. There is growing interest in defining these unusual parasite-synthesized glycan antigens and using them to understand immune responses, their roles in immunomodulation, and in using glycan antigens as potential vaccine targets. A key problem in this area, however, has been the lack of information about the enzymes involved in elaborating the complex repertoire of glycans represented by the schistosome glycome. Recent availability of the nuclear genome sequences for *Schistosoma* sp. has created the opportunity to define the glycogenome, which represents the specific genes and cognate enzymes that generate the glycome. Here we describe the current state of information in regard to the schistosome glycogenome and glycome and highlight the important classes of glycans and glycogenes that may be important in their generation.

Keywords: glycans, glycoconjugates, genome, glycosyltransferases, glycan biosynthesis, schistosomiasis

INTRODUCTION

Schistosomiasis is a debilitating vascular disease caused by an infection with parasitic helminths of the *Schistosoma* species. It is a major public health concern in many developing countries with a wide range of clinical manifestations (Cummings and Nyame, 1996; Jang-Lee et al., 2007; Savioli and Daumerie, 2010). These parasitic worms have a complex life cycle that alternates between an intermediate mollusk host and a definitive vertebrate host resulting in significant morbidity and mortality for the infected human or animal. With millions of people afflicted worldwide in over seventy tropical and subtropical countries, the World Health Organization (WHO) considers schistosomiasis second in socioeconomic importance among diseases worldwide and the third most important parasitic disease in terms of public health impact (Cummings and Nyame, 1996; Savioli and Daumerie, 2010; Elbaz and Esmat, 2013).

Despite years of research on schistosome biology, millions are still affected and at risk due to insufficient prevention, diagnostics, treatments, and absence of a vaccine. Previous vaccine platforms have failed because of the complex tissue architecture of schistosomes and a lack of innovative strategies to protect against complex, multicellular pathogens. The major immune response to schistosome infection is directed to carbohydrate (glycan) antigens in surface and secreted glycoproteins and glycolipids (Omer-Ali et al., 1986, 1989; Eberl et al., 2001; Kariuki et al., 2008). Schistosomes possess an abundance of complex and unique glycans and glycoproteins that interact with both the innate and adaptive arms of immunity in human and animal hosts in a variety of ways (reviewed by Prasanphanich et al., 2013). A major limitation in the study of glycans is that we are currently unable to chemically synthesize them in an affordable and facile manner. It is also not feasible to isolate significant quantities of individual glycans from the parasites at each developmental stage. In the past several years, the availability of genomic databases has allowed us and others to take an alternative approach using enzyme technology in a chemo-enzymatic approach to generate glycans and explore their recognition by antibodies and glycan-binding proteins (Kupper et al., 2012; Peng et al., 2012; Ban et al., 2013; Tefsen and van Die, 2013; Luyai et al., 2014; Prasanphanich et al., 2014). In this review, we will discuss specific components of the schistosome glycome that contribute to immune responses and identify key *Schistosoma* genes involved in glycan synthesis. Defining the glycogenome of schistosomes will aid our understanding of the significance and breadth of the immune response to glycan antigens, as well as provide a platform for future diagnostic and vaccine developments.

IMPORTANCE OF SCHISTOSOME GLYCOCONJUGATES

Schistosomes, like other parasitic helminths, produce many complex carbohydrate structures linked to proteins and lipid, including N-glycans, O-glycans, and glycolipids, which are structurally distinct from their definitive host. It has long been accepted that glycans and glycoconjugates play an essential role in the biology of the parasite, in particular with regard to host-pathogen interactions, however their specific functions remain unclear (reviewed by Cummings and Nyame, 1996, 1999; Hokke et al., 2007; Prasanphanich et al., 2013). Unlike the sequence of a protein, in which homologous protein sequences among species imply homologous functions, glycan sequences are more complex and seemingly slight changes in structures can profoundly affect biological activities in unpredictable ways. Over the past few decades researchers have found that schistosomal glycans

are bioactive and can induce innate and adaptive immunological responses (Hokke and Yazdanbakhsh, 2005; Van Die and Cummings, 2006, 2010; Meevissen et al., 2012a; Van Diepen et al., 2012b). Circulating antigens have also proven useful as diagnostics in human and animal hosts (Nyame et al., 2004; Van Dam et al., 2004; Sousa-Figueiredo et al., 2013). A deeper understanding of these glycans and glycoconjugates, and their ability to modulate the immune system, could potentially ignite innovative new strategies for lessening the mortality and morbidity caused by these parasites.

HOST-PARASITE INTERFACE

The surface of the schistosome, as well as secreted and excreted products, are rich in glycans linked to proteins and lipids and serve as the main source of parasite-host interactions. The schistosome surface is complex and poorly understood, and the expression of surface proteins and glycans is highly variable throughout its life stages (Simpson et al., 1984; Robijn et al., 2005; Braschi et al., 2006). Unlike nematodes, which are protected by a cuticle, schistosomes are covered by a syncytial layer of cells called the tegument. The tegument is comprised of secreted lipid-rich membranocalyx and glycan-rich glycocalyx, which includes membrane, secreted glycoconjugates, and associated materials. While the glycocalyx is partially lost upon transformation of cercariae to schistosomules, it remains clearly prominent in adult worms (Samuelson and Caulfield, 1985; Dalton et al., 1987; Abou-Zakham et al., 1990; Kusel et al., 2007).

The role of glycans in host-parasite interactions during snail infection is less understood. Evidence suggests that glycoconjugates might play a pivotal role in both cellular and humoral immune interactions between their molluscan intermediate hosts miracidia and sporocytes (Cummings and Nyame, 1996; Loker and Bayne, 2001; Yoshino et al., 2001; Nyame et al., 2002; Peterson et al., 2009). Fucosylated structures prominently expressed on the larval surface and amongst glycoproteins released during larval transformation and early sporocyst development indicate a role for these glycan epitopes in snail–schistosome interactions. Also, snail hosts share some glycans with schistosomes suggesting an evolutionary convergence of carbohydrate expression between schistosomes and their snail host (Castillo et al., 2007; Lehr et al., 2008; Peterson et al., 2009; Yoshino et al., 2013, 2012).

IMMUNE MODULATION

Prior studies in the field of parasitology suggested the glycans of parasitic worms resembled those of their vertebrate hosts, leading to a concept of molecular mimicry (Damian, 1964). However, modern studies of schistosomes and other helminth glycoconjugates show that the glycans generated by these organisms are unique and generally have features very unlike those of vertebrate hosts (reviewed by Van Diepen et al., 2012b; Prasanphanich et al., 2013). These observations, as well as the evidence that parasite-derived glycans are bioactive as well as immunogenic, have led to the concept of glycan gimmickry, which highlights the key roles of parasite glycans in immunomodulation and evasion of host responses and is an alternative model to pathogenic molecular mimicry (Van Die and Cummings, 2010). Schistosome glycans lack the most common mammalian terminal sugar, sialic acid, which is found in both glycoproteins and glycolipids of all vertebrate cells. Additionally, as we will discuss, schistosome N- and O-glycans often contain poly-fucose and xylose, which are glycan modifications not found in vertebrate glycans (Faveeuw et al., 2003; Geyer et al., 2005; Paschinger et al., 2005a; Meevissen et al., 2012b; Luyai et al., 2014).

It has long been recognized that schistosome glycans, and other helminth glycans, harbor potent immunomodulatory properties and have been found to induce innate and adaptive immune responses in the host (Thomas and Harn, 2004; Hokke and Yazdanbakhsh, 2005; Ju et al., 2006; Van Die and Cummings, 2006; Hokke et al., 2007). Understanding this process could translate to improved outcome of disease and co-infections, as well as aid in the development of anti-schistosome vaccines (Bergquist and Colley, 1998; Knox and Redmond, 2006; Mcmanus and Loukas, 2008). Parasite molecules involved in skewing toward a Th2 environment and down-regulation of the immune response could be potential treatments for autoimmune or inflammatory conditions. There has already been success in treating animal models of type-1 diabetes, colitis, and multiple sclerosis with therapeutic helminthic infection (Zaccone et al., 2003; La Flamme et al., 2004; Smith et al., 2007).

For example, the Lewis X (Le^X) trisaccharide, a common glycan motif in schistosome eggs, is a potent inducer of the Th2 responses often via recognition by Toll-like receptors (TLRs) and C-type lectin receptors (Okano et al., 1999, 2001; Velupillai et al., 2000; Thomas et al., 2003, 2005; Van Die et al., 2003; Atochina and Harn, 2005). In fact, egg antigens can suppress TLR-induced DC activation when internalized by a combination of DC-SIGN, MR, and/or MGL (Van Liempt et al., 2007). Le^X can also induce proliferation of B cells, the production of suppressive cytokine IL-10 in peripheral blood mononuclear cells, and function as an initiator and/or modulator of granuloma formation (Velupillai and Harn, 1994; Velupillai et al., 2000).

DIAGNOSTIC MARKERS AND ANTI-GLYCAN ANTIBODIES

Schistosomiasis is routinely diagnosed by the presence of eggs in the stool or urine, depending on the infecting strain. However, eggs are not consistently shed, the severity of infection (worm burden) cannot be accurately determined from egg count, and false negatives are still common (Booth et al., 2003; Gryseels et al., 2006; Utzinger and Keiser, 2008; Knopp et al., 2011). Carbohydrates as diagnostic antigens might be a superior alternative. Assays detecting circulating cathodic antigen (CCA) and circulating anodic antigen (CAA) in serum or urine appear to be more reliable and sensitive diagnostic methods since levels of these antigens fluctuate less than egg counts (Polman et al., 1998). There is now a commercially available CCA dipstick test that successfully detects infections in very young children and is showing promise in point-of-care settings, and a dry format assay which rapidly detects CAA in serum (Stothard et al., 2011; Sousa-Figueiredo et al., 2013; Van Dam et al., 2013, 2004).

Anti-glycan antibodies, which dominate the humoral response, are also being considered for diagnostic purposes. Certain defined glycans including LDN, Le^X, F-LDN, and LDN-DF have different, stage-specific antibody binding profiles when used to probe worm antigen (Eberl et al., 2001; Van Remoortere

et al., 2001, 2003; Naus et al., 2003; Nyame et al., 2003; Hokke et al., 2007). Other highly fucosylated epitopes, such as F-LDN-F and DF-LDN-DF are possible diagnostic epitopes due to their unique expression on schistosomes. The monoclonal antibody 114-4D12, which targets DF-LDN-DF, can detect unconjugated oligosaccharides excreted from *S. mansoni* eggs in infected urine. MS/glycan based studies may lead to a new egg-load-related assay helpful in the detection of mild infections (Robijn et al., 2007, 2008). However, given the differential responses to discrete glycans it is unclear whether immunodiagnostic tools could differentiate between current and past infection.

GLYCOME APPROACHES AND LIMITATIONS

The identification and sequencing of schistosome glycans began in the 1980's with the identification of unusual N- and O-glycans synthesized by short-term cultures of schistosomula and adult worms (Nyame et al., 1987, 1988a,b, 1989; Makaaru et al., 1992; Srivatsan et al., 1992a,b). Subsequent studies (Bergwerff et al., 1994; Van Dam et al., 1994; Khoo et al., 1995, 1997a,b; Frank et al., 2012) (also see reviews by Cummings and Nyame, 1996, 1999; Hokke and Deelder, 2001; Hokke and Yazdanbakhsh, 2005; Hokke et al., 2007; Prasanphanich et al., 2013) identified complex types of glycan structures in both membrane associated and circulating antigens. These types of studies, now generally recognized as *structural glycomics*, involve complex analyses incorporating tandem mass spectrometry (MS), nuclear magnetic resonance (NMR) and compositional and linkage analyses. Unfortunately, while the field has advanced tremendously in identifying many types of glycans synthesized by schistosomes and even glycan structure differences between sexes and schistosome species, it is likely that only a tiny fraction of the total set of glycans synthesized by any stage of the parasite is known (Khoo et al., 1997a; Nyame et al., 1998, 2000; Van Die et al., 1999; Wuhrer et al., 2006b). Thus, much remains to be learned about the specific sequences and complete structures of schistosome glycans as well as their temporal and spatial expression. One obvious limitation to these studies is that schistosomes are parasites and must be isolated from infected animals, thus limiting their availability as well as creating potential problems in contamination by glycans from the hosts. While structural studies remain important for confirming hypothesized structures and characterizing glycan-protein interactions, a genome method provides many advantages.

A GENOMIC APPROACH

While knowledge of schistosome glycans is woefully incomplete, the available evidence indicates that many different glycan linkages and sequences occur. In both simpler organisms, such as *C. elegans*, as well as more complex organisms, such as mice and humans, many genes within the genome have been shown to encode enzymes responsible for elaboration of the glycome. These genes, typically referred to as comprising the glycogenome, encode glycosyltransferases, glycosidases, sugar and nucleotide sugar metabolizing enzymes important in glycan biosynthesis, nucleotide sugar transporters, and glycan-binding proteins. It is estimated that mice and humans have over 900 genes involved in elaboration and recognition of their glycomes (Cummings and Pierce, 2014). This background knowledge has set the stage for now exploring the glycogenomes of schistosomes and other parasites and identifying the genes important for elaboration of their glycomes (**Figure 1**).

In 2009 the nuclear genome of *S. mansoni* was published in *Nature* as a result of a successful international collaboration among multiple institutions (Berriman et al., 2009). The analysis of the 363 megabase genome utilized several gene prediction algorithms, including the extended similarity group (ESG) method, which performs iterative sequence database searches and annotates a query sequence with Gene Ontology terms. At least 11,809 genes were annotated encoding over 13,000 transcripts with unusual intron sizes, distributions, and frequent alternative splicing. The annotated genome sequence was submitted to EMBL (accession numbers FN357292-FN376313) and GeneDB (http://www.genedb.org/Homepage/Smansoni) (Berriman et al., 2009; Chitale et al., 2009; Criscione et al., 2009).

Shortly after the genome was published, SchistoDB (http://schistoDB.net/) was created to offer researchers a plethora of tools for genomic data mining. SchistoDB incorporates sequences and annotations for *S. mansoni* in a single directory. Several genomic scale analyses are available as well as expressed sequence tags, oligonucleotides, and metabolic pathways. By 2012, the directory was expanded by integrating the data sets from other *Schistosome* species, *S. japonicum* and *S. haematobium* (Zerlotini et al., 2009; Zhou et al., 2009; Young et al., 2012). Current studies have utilized the genomic data to highlight transcriptional differences seen throughout lifecycle progression and identify anti-schistosomal candidate molecules including fucosyltransferases via transcriptome analyses and gene micro-arrays (Fitzpatrick et al., 2009; Protasio et al., 2012).

The KEGG (Kyoto Encyclopedia of Genes and Genomes) database uses large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies to help scientists understand high-level functions and utilities of various biological systems. With the information generated from the *Schistosoma* genome sequences, KEGG Glycan constructed pathway maps on molecular interactions including glycan biosynthesis and metabolism that are annotated with the specific enzymes/proteins involved and the corresponding genes (http://www.genome.jp/kegg/glycan). The system also characterizes gene/protein functions across organisms, allowing for genes like glycosyltransferases to be finely classified within ortholog groups which may have been overlooked by previous sequence similarity algorithms (Aoki et al., 2004; Kawano et al., 2005; Hashimoto et al., 2006, 2009; Kanehisa et al., 2010).

With the amount of information now available, genomics technologies can be applied to unravel the biology of some of these parasites, including the complexity of glycan biosynthesis (**Figure 1**). Given the vast assortment of glycan epitopes, as well as available databases, it can be predicted that schistosomes express a plethora of glycosyltransferases and other genes required for glycan biosynthesis (**Table 1**). A more thorough understanding of the schistosome glycome could promise faster identification of targets for diagnostics and drug development, as well as a collaborative approach to antigen chemo-enzymatic synthesis and discovery of a glycan-based vaccine platform.

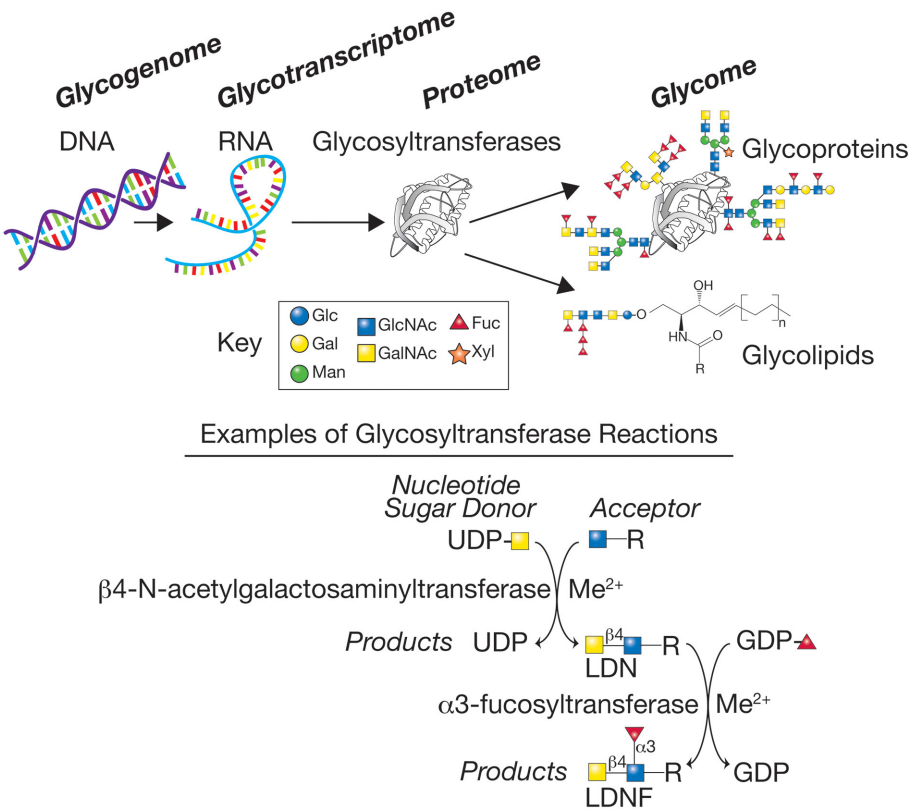

FIGURE 1 | The glycogenome represents the genes encoding the various glycosyltransferases, glycosidases, sugar, and nucleotide sugar metabolizing enzymes important in glycan biosynthesis, and nucleotide sugar transporters. The glycosyltransferases generated from the glycotranscriptome in schistosomes represent a large class of predicted enzymes, often requiring metal cofactors, such as manganese (Me^{2+}), that synthesize glycans using donor nucleotide sugars to form glycosidic bonds to acceptors, here represented by a sugar-R, where R = sugar, protein, or lipid to which a sugar is linked. The products of the biosynthetic reactions have specific glycosidic linkages, e.g., β1,4 or α1,3, and the glycans produced are often acceptors for additional enzymes, thus generating the complex set of glycans representing the glycome of the organism. Examples are shown for two glycosyltransferase reactions that together can synthesize the LDN and LDNF antigen determinants. The key for several of the monosaccharides found in schistosome glycans are indicated—Glc (Glucose), Gal (Galactose), Man (Mannose), GlcNAc (N-acetylglucosamine), GalNAc (N-acetylgalactosamine), Fuc (Fucose), and Xyl (Xylose).

GLYCAN BIOSYNTHESIS PATHWAYS

Previous structural studies of schistosome glycoconjugates primarily depend on analytical techniques, but are limited due to insufficient quantities of glycans and the need to prepare glycans from parasites isolated from infected hosts, as well as variation in glycan expression among the life stages, resulting in incomplete glycome profiling (Khoo et al., 2001; Paschinger et al., 2005a; Van Balkom et al., 2005; Wuhrer et al., 2006a,b; Hokke et al., 2007; Roger et al., 2008). Nevertheless, using the available glycan sequence data and developmentally-regulated expression of glycan antigens, it is predicted that schistosomes contain a multitude of different classes of glycosyltransferases involved in glycan biosynthesis and that their expression is differentially regulated by tissue and life stage (Joziasse, 1992; Breton et al., 1998; Kapitonov and Yu, 1999). To date, very few of these enzymes in distinct glycan classes have been studied in detail, however, with the genomic data now available, glyco-related genes might be easier to explore in the future (**Figure 1**; **Table 1**).

N-GLYCANS

The N-glycans found in *Schistosoma* glycoproteins feature high mannose and complex-type structures common in eukaryotes and higher organisms (Nyame et al., 1988a, 1989). Thus, it appears that schistosomes follow the conventional pathway for N-glycan core synthesis, where the precursors are synthesized on the cytoplasmic face of the ER membrane beginning with dolichol phosphate (Dol-P) in a step-wise process catalyzed by *ALG* gene enzymes (for *al*tered in *g*lycosylation). Fourteen sugars are sequentially added before en bloc transfer of the entire structure to an Asn-X-Ser/Thr site in a protein. The protein-bound N-glycan is subsequently remodeled in the ER and Golgi by a complex series of reactions catalyzed by membrane-bound glycosidases and glycosyltransferases (Sharma et al., 2005; Stanley et al., 2009).

The genome of *S. mansoni* appears to contain homologs to the ALG genes required for synthesis and remodeling (**Table 1**). The splice variant Smp 051360.1 most likely functions as a UDP-N-acetylglucosamine (GlcNAc) dolichylphosphotransferase which

Table 1 | Components of the *S. mansoni* glycogenome.

Category	# of Putative Genes	Gene ID		
GLYCOSYLTRANSFERASES				
Galactosyltransferases and N-acetylglucosaminyltransferases (GalTs and GnTs)[a]	14[b]	Smp 058670	Smp 056260	Smp 102400
		Smp 006930	Smp 024650	Smp 007950
		Smp 210290[c]	Smp 015920	Smp 151210
		Smp 146430	Smp 153110	Smp 151220
		Smp 042720	Smp 149820[c]	
N-acetylgalactosamine transferases (GalNAcTs)	7[b]	Smp 057620[c]	Smp 159490[c]	Smp 005500[c]
		Smp 139230[c]	Smp 211240	Smp 021370
		Smp 047240		
Fucosyltransferases (FucTs)	22[d]	Smp 175120	Smp 175120	Smp 137740
		Smp 194990	Smp 205640	Smp 028910
		Smp 199790	Smp 154410	Smp 065240
		Smp 030650	Smp 138750	Smp 212520
		Smp 138730	Smp 211180	Smp 137730
		Smp 193870	Smp 193620	Smp 142860
		Smp 054300	Smp 209060	Smp 129750
Xylosyltransferase	2	Smp 128310	Smp 125150	
BIOSYNTHESIS PATHWAYS				
N-Glycan	18[e,f]	Smp 051360	Smp 045430	Smp 082710
		Smp 055010	Smp 177080	Smp 052330
		Smp 161590	Smp 103930	Smp 020770
		Smp 035470	Smp 055200	Smp 105680
		Smp 210360	Smp 210370	Smp 024580
		Smp 018760	Smp 018750	Smp 143430
O-Glycan	5[e]	Smp 149820[c]	Smp 057620[c]	Smp 015949[c]
		Smp 005500[c]	Smp 139230[c]	
Glycolipid	2[e]	Smp 160210	Smp 157080	
GPI-anchor	14[e]	Smp 154600	Smp 136690	Smp 145290
		Smp 155890	Smp 155900	Smp 017730
		Smp 046880	Smp 163640	Smp 152460
		Smp 035080	Smp 128810	Smp 177040
		Smp 053460	Smp 021980	
GAG	6[e]	Smp 178490	Smp 083130	Smp 124020
		Smp 075450	Smp 134250	Smp 210290[c]

[a] *Grouped in database, see text for details.*

[b] *Tally in text references a subset of genes (Ex: 3 β1-4GalNAcTs, 7 total GalNAcTs)*

[c] *Listed in both glycosyltransferases and pathways.*

[d] *Genes have redundancies, see text reference for details.*

[e] *Denotes current gene annotations discussed in the text. Not an exhaustive list.*

[f] *Tally does not account for splice variants.*

forms GlcNAc-P-P-Dol. A second GlcNAc and five mannose (Man) residues are subsequently added by specific glycosyltransferases to generate $Man_5GlcNAc_2$-P-P-Dol on the cytoplasmic side of the ER. Homologs in this pathway include Smp 045430.3 and Smp 082710 as UDP-N acetylglucosaminyltransferase (GlcNAcT) subunits (similar to ALG 14), Smp 055010 as a chitobiosyldiphosphodolichol α-mannosyltransferase, Smp 177080 as an α-1,3-mannosyltransferase (ALG 2), and Smp 052330 probably functions like asparagine-linked glycosylation protein 11 (ALG 11). Other genes responsible in forming the common 14-sugar lipid-linked precursor in animals, $Glc_3Man_9GlcNAc_2$-P-P-Dolichol, are Smp 161590 (simply designated a glycosyltransferase but contains regions similar with an α-1,6-mannosyltransferase), splice variants of

Smp 103930 (α-1,2-mannosyltransferase), and Smp 096910/Smp 15120 (α-1,3-glucosyltransferases) (Sharma et al., 2005; Berriman et al., 2009; Stanley et al., 2009).

The transfer of the 14-sugar glycan in $Glc_3Man_9GlcNAc_2$-P-P-Dolichol to Asn-X-Ser/Thr sequons of a newly synthesized protein is catalyzed by a set of proteins termed the oligosaccharyltransferase (OST) complex. *S. mansoni* genes likely to function as OST subunits are Smp 020770 (α unit), Smp 035470 (β unit), Smp 055200 (γ unit), Smp 105680 (ribophorin I), and Smp 210360/210370 (δ unit) (Chavan et al., 2005; Berriman et al., 2009; Stanley et al., 2009). After covalent attachment of the 14-sugar glycan ($Glc_3Man_9GlcNAc_2$-Asn) a series of processing reactions trim the glycan using α-glucosidases. Smp 024580 and Smp 018760 most likely remove the three Glc residues leaving the high mannose $Man_9GlcNAc_2$-Asn structure. Smp 018750 (α-1,3-mannosidase) and Smp 143430 (α-mannosidase II) remove mannose allowing for the N-glycans to be recognized and further extended/modified by glycosyltransferases, as discussed below, which generate the hybrid or complex-type N-glycans with terminal glycan motifs (Nyame et al., 1988a, 1989; Wuhrer et al., 2006b; Berriman et al., 2009; Stanley et al., 2009).

O-GLYCANS

O-glycosylation in schistosomes range from a single sugar residue to large, complex, multi-fucosylated structures fluctuating from 12 to at least 60 glycosyl residues in length in the cercarial glycocalyx (Nyame et al., 1987, 1988b; Khoo et al., 1995). Many surface localized schistosome glycoproteins contain a simple O-linked GlcNAc, which probably occurs on intracellular and intranuclear glycoproteins (Nyame et al., 1987; Ma and Hart, 2014). Other common structures include Galβ1-3(Galβ1-6)GalNAc (O-glycan schisto core) and mucin-type sequences including GalNAcα1-Ser/Thr (Tn antigen), Galβ1-3GalNAcα1-Ser/Thr (T antigen, core 1), and Galβ1-3(GalNAcβ1-6)GalNAc (core 2) with the core 1 structure being the most common (Nyame et al., 1988b; Van Dam et al., 1994; Jang-Lee et al., 2007). The more complex O-glycans contain unique repeating elements with GalNAcβ1-4GlcNAcβ1-3Galα1-3 units carrying fucosylated sequences linked to the internal GlcNAc and terminal GalNAc structures (Nyame et al., 1987; Cummings and Nyame, 1996).

In vertebrates, the core 1 O-glycan disaccharide is also the most common of such O-glycan cores and is a precursor to more complex O-glycans such as extended core 1 and core 2 structures. The core 1 structure is synthesized from GalNAcα1-Ser/Thr by the addition of galactose, a reaction catalyzed by the enzyme core 1 UDP-Gal:GalNAcα1-Ser/Thr β1,3-galactosyltransferase (core 1 β3-Gal-T or T-synthase) (Wandall et al., 1997; Ju and Cummings, 2002; Ju et al., 2006). In *S. mansoni*, Smp 149820 is the only gene designated a glycoprotein-N-acetylgalactosamine β3galactosyltransferase and is considered the ortholog to T-synthase (Ju and Cummings, 2002), whereas *S. japonicum* has five genes annotated as core 1 β3-Gal-transferase (Sjp 005210, Sjp 0042730, Sjp 0055580, Sjp 0064840, Sjp 0093870) (Berriman et al., 2009; Zhou et al., 2009). The gene in the nematode *C. elegans* encoding the T-synthase was identified earlier to encode a functional enzyme that also has homology to the *S. mansoni* gene Smp 149820 (Ju et al., 2006).

Several UDP-*N*-Acetylgalactosamine:polypeptide*N*-acetylgalactosaminyltransferases (GalNAc-transferases, ppGalNAcTs), which generate GalNAcα1-Ser/Thr have been identified and characterized in humans. While the human ppGalNAcTs show similarities in domain structures, sequence motifs, and conserved cysteine residues the overall amino acid sequence similarity of less than 50% suggests changes within this enzyme family during evolution (Wandall et al., 1997). The *S. mansoni* ppGalNAcTs (Smp 005500, Smp 057620, Smp 139230, and Smp 159490) have comparable levels of amino acid similarity (approximately 30–50%) among them (Berriman et al., 2009).

GLYCOLIPIDS

Schistosome glycolipids consist of galactosylceramide, glucosylceramide, and glycolipids with extended glycans emanating from the "schisto core" (GalNAcβ1-4Glc-ceramide). This is in contrast to the human glycolipid core, which is lactosylceramide Galβ1-4Glc-ceramide. Schistosomes synthesize glycosphingolipids with a similar acceptor to vertebrates using a glucocerebroside precursor, but instead of adding the galactose, as in animals, schistosomes instead generate the "schisto-core" structure by the addition of a β1-4GalNAc residue (Makaaru et al., 1992; Wuhrer et al., 2000). The simple schisto-core structure is extensively modified in egg glycosphingolipids of *S. mansoni* and *S. japonicum* with repeating GlcNAc motifs with multiple fucosylation units (Fucα1-2Fucα1-3GlcNAcβ1-R) (Khoo et al., 1997a; Cummings and Nyame, 1999). *S. mansoni* glycolipids are dominated by fucose. Cercariae often express terminal Le^X and pseudo Lewis Y (Fucα1-3Galβ1-4(Fucα1-3)GlcNAc; pseudoLe^y) structures, while the Fucα1-3GalNAc terminal element was confirmed in *S. mansoni* egg glycolipids (Wuhrer et al., 2000, 2002).

Sequencing of the *S. mansoni* genome indicated that schistosomes contain a full complement of genes required for most lipid metabolic processes. In reference to ceramide as a major precursor to glycosphingolipids, *S. mansoni* encodes two putative ceramide glucosyltransferases (Smp 160210 and Smp 157080) while *S. japonicum* genome contains four (Sjp 0094210, Sjp 0065630, Sjp 0054080, Sjp 0093880) (Berriman et al., 2009; Zhou et al., 2009). Although not a "classical" sugar, the genome sequencing of *S. mansoni* also revealed a lipid deficiency where the worms must depend on its host as a source of inositol (Brouwers et al., 1997; Berriman et al., 2009).

GPI-ANCHORED GLYCOPROTEINS

It is well known that S. *mansoni* and other schistosome species produce glycoproteins anchored to membranes through a glycosylphosphatidylinositol lipid anchor (GPI anchor) and thus lack a transmembrane protein domain. Such GPI anchored glycoproteins have now been found in all animal cells, and in the parasite world were first extensively studied in trypanosomes (reviewed by Ferguson, 1999). Examples of common GPI-anchored proteins previously characterized in schistosomes include alkaline phosphatases and acetylcholinesterase (Espinoza et al., 1988; Sauma et al., 1991; Hawn and Strand, 1993; Castro-Borges et al., 2011). Both *S. mansoni* and *S. japonicum* genomes contain annotations for acetylcholinesterase (Smp 154600, Smp 136690, Sip 0070510, Sjp 0045440, and Sjp 0036280), however only *S. mansoni*

appears to have genes currently designated as alkaline phosphatases (Smp 145290, Smp 155890, and Smp 155900) (Berriman et al., 2009; Zhou et al., 2009). *S. mansoni* also expressed a 200 kDa GPI-anchored glycoprotein on its surface which is a target for antibodies that can act synergistically with praziquantel treatment (Sauma et al., 1991; Hall et al., 1995). According to the database this protein is a product of the gene Smp 017730, however that record has not yet been subjected to final NCBI review (Berriman et al., 2009). Vaccination with *S. mansoni* tegumental GPI-anchored glycoproteins partially protected mice from infection and reduced infection, warranting further investigation of the biochemistry and genetics of such glycoconjugates in schistosomes (Martins et al., 2012).

Previously, details about the GPI-anchor biosynthesis pathway in schistosomes were unknown, however several putative proteins from the *S. mansoni* genome are believed to be involved. Phosphatidylinositol N-acetylglucosaminyltransferase catalyzes the first step of GPI anchor formation in all eukaryotes. In mammalian cells, this enzyme is composed of at least five subunits (PIG-A, PIG-H, PIG-C, GPI1, and PIG-P), with PIG-A functioning as the catalytic subunit (Hawn and Strand, 1993; Watanabe et al., 1998). A splice variant of Smp 046880 (termed Smp 046880.1) has around 50% identity with PIG-A isoforms in a variety of mammals. Smp 163640 and Smp 152460 also show homology with subunits PIG-P and GPI1 respectively. N-acetylglucosaminylphosphatidylinositol deacetylase (PIG-L), the enzyme responsible for the second step in GPI-anchor formation, and PIG-M, which transfers the first mannose to glycosylphosphatidylinositol on the lumenal side of the ER also show homology with the products from genes Smp 035080 and Smp 128810 (Nakamura et al., 1997; Maeda et al., 2001; Berriman et al., 2009). Other genes possibly involved in building the common GPI ethanolamine-glycan core include Smp 177040, Smp 053460, and Smp 021980. There is a probability that schistosomes also encode enzymes which allow for heterogeneity within the common core of GPI-anchors, like what is observed in mammals (Takahashi et al., 1996; Kang et al., 2005; Berriman et al., 2009; Ferguson et al., 2009).

GLYCOSAMINOGLYCANS AND PROTEOGLYCANS

Little is known about the glycosaminoglycan (GAG) or proteoglycan (PG) content of schistosomes. Two studies have isolated GAGs from schistosomes, demonstrating the presence of glycans resembling heparin/heparan sulfate (HS), chondroitin sulfate (CS) and hyaluronic acid (Robertson and Cain, 1985; Hamed et al., 1997). It has been hypothesized that heparin/heparan sulfate in the worm tegument could provide a mechanism of immune evasion by inhibiting the host clotting cascade; however, it has not been verified whether the GAGs isolated are from the parasite or the host and their structures have not been chemically defined (Robertson and Cain, 1985).

The *Schistosoma* genomes indicate that much of the genetic machinery necessary for synthesizing GAGs is present. *S. mansoni*, *S. japonicum*, and *S. haematobium* all have genes homologous to the xylosyltransferase genes in mammals, mollusks, and nematodes which code for protein-O-xylosylation activity (XYLT1 and XYLT2 in mammals; XYLT or *sqv8* in *C. elegans*). These genes encode enzymes which catalyze the first step in addition of the HS/CS core to proteoglycans, and share the conserved Xylosyltransferase C terminal domain and other domains with the Core-2/I-branching enzyme family. Other enzymes necessary for construction of the HS/CS core that have been characterized in *C. elegans* include *sqv3* (Gal-transferase I in mammals, encoded by β4GalT7), *sqv8* (GlcA transferase I) and *sqv7* (a UDP-GlcA/GalNAc transporter) (Bulik et al., 2000). The three *Schistosoma* genomes possess genes homologous to each of these, containing the relevant conserved domains (B4GALT7: Smp 210290, Sjp 0062870, Sha 200402; UDP-GlcA/GalNAc transporter: Smp 178490; Sjp 0089300, Sha 103448; GlcA transferase I: Smp 083130, Sjp 0062810, Sha 108192). The enzymes that catalyze polymerization of HS chains in vertebrates are exotosins (EXTs), at least three of which are annotated for *S. mansoni* (Smp 172060, Smp 146320—two splice variants, Smp 073220). Putative HS 2-O- and 6-O-sulfotransferases and a HS N-deacetylase/N-sulfotransferase are also annotated (Smp 124020, Smp 075450, Smp 134250; Sjp 0060410, Sjp 0082020, Sjp 0094660) (Berriman et al., 2009; Zhou et al., 2009). Interestingly, no homolog of 3-O-sulfotransferase, the activity of which is required for generating the anti-thrombin inhibitory motif of mammalian HS, was found (Ragazzi et al., 1987).

Circulating anodic antigen (CAA) is another GAG-like, O-linked glycoprotein antigen excreted by schistosomes, which is also under investigation as a diagnostic target (Vermeer et al., 2003). CAA is completely unique among all previously identified glycan structures, consisting of the repeating trisaccharide GalNAcβ1,6-(GlcAβ1,3)-GalNAcβ1,6-, although it slightly resembles the backbone sequence of mammalian chondroitin sulfate, a repeating disaccharide containing GalNAc and GlcA (Deelder et al., 1980; Bergwerff et al., 1994; Esko et al., 2009). Currently, there are no genes annotated as β-1,6-GalNAcT in the *Schistosoma* genomes.

Interestingly, the NCBI gene database contains a second gene annotated as a β3GlcAT (Accession no. CAD98790.1) (Zhou et al., 2009). The conserved residues and domains of β3GlcAT responsible for donor (UDP-GlcA) and acceptor (UDP-Gal) binding, and other critical aspects of the enzyme function, have been characterized (Fondeur-Gelinotte et al., 2006). The residues associated with donor binding are well-conserved in the schistosome genes. The conserved amino acids associated with acceptor binding are almost completely maintained among the human, mouse, *C. elegans*, *S. mansoni*, and Sjp 0062810 β3GlcAT genes. However, there is a 15-amino acid stretch within the acceptor binding region in which all the sequences are well-conserved except for the second *S. japonicum* β3GlcAT gene. It is tempting to speculate that if the second β3GlcAT indeed represents a distinct gene sequence, then it may be responsible for the addition of GlcA to CAA, a linkage that is otherwise unknown in the animal kingdom. Or, perhaps one of the EXT genes or splice variants could be involved in CAA synthesis.

GLYCAN MOTIFS

It should be noted that sialic acids, common terminal sugars of mammalian glycans, have never been demonstrated as part of schistosome glycan motifs (Nyame et al., 1987, 2004). In animals

and microbes, sialic acid must be activated for use in glycan biosynthesis by conjugation with CTP, a process catalyzed by CMP-Sialic acid synthetase (Kean et al., 2004). These are encoded by the CMAS gene, which is highly conserved among vertebrates and well-conserved even in other prokaryotes and eukaryotes (Sellmeier et al., 2013). No genes with significant homology spanning the functional domains of this gene were found in *C. elegans* or *Schistosoma* genomes.

LN AND LDN

LacNAc (Galβ1-4GlcNAc; LN; *N*-acetyllactosamine) and LacdiNAc (GalNAcβ1-4GlcNAc; LDN) are terminal modifications in *Schistosoma* glycoproteins. LN is more typically found in mammalian glycan structures and is frequently modified through sialylation, fucosylation, sulfation, or other sugars to generate a wide range of glycan epitopes. Glycans containing the LDN motif are commonly expressed by many invertebrates, including schistosome intermediate hosts and human pathogens, but also sometimes occur in vertebrates including several mammalian glycoproteins (Khoo et al., 1997a; Van den Eijnden et al., 1997; Van de Vijver et al., 2006; Van Die and Cummings, 2010; Yoshino et al., 2012, 2013). LDN determinants present in parasite glycans have been shown to generate a humoral response by the human immune system, and interestingly both LN and LDN expression can initiate the formation of a granuloma in humans (Van Remoortere et al., 2001; Van de Vijver et al., 2006; Prasanphanich et al., 2014).

Galactosyltransferases (GalTs) and N-acetylgalactosaminyl transferases (GalNAcTs) are crucial to LN and LDN synthesis, respectively. The presence of β1-4GalNAcT and β1-4GalT activity were discovered using extracts created from *S. mansoni* and the bird schistosome *Trichobilharzia ocellata* (Rivera-Marrero and Cummings, 1990; Neeleman et al., 1994; Srivatsan et al., 1995). Unlike its mammalian homolog, the schistosome β1-4GalT activity is not altered by the presence of α-lactalbumin (Sato et al., 1998). While a family of human glycosyltransferases responsible for LN synthesis has been reported, the first β1-4GalNAcT cloned and characterized was from *C. elegans* (Wandall et al., 1997; Amado, 1999; Kawar et al., 2002). The Ceβ1-4GalNAcT has been shown to be fully functional with the ability to create the LDN antigen on transfected Chinese Hamster Ovary cells (Kawar et al., 2002). An equivalent enzyme that creates the UDP-Gal:β-1,4-GlcNAc linkage necessary for the LN structure has not been identified in *C. elegans*. These advancements in understanding glycosyltransferases are a necessary first step, but research is still far from understanding the complex regulation and glycomics of LN and LDN synthesis.

Currently the schistosome database contains several glycosyltransferases potentially capable of generating these glycan linkages. A search of the database yields three putative β1-4GalNAcT and six β1-4GalT sequences (Berriman et al., 2009). The nucleotide sequences of the β1-4GalNAcTs contain little homology to the *C. elegans* equivalents. However, protein alignments show improved homology among the catalytic domains of the *S. mansoni* and *C. elegans* β1-4GalNAcTs with approximately 30–40% identity. Similar levels of homology are found when comparing the Ceβ1-4GalTs to the putative β1-4GalT sequences. However, the database is far from complete, with many gene sequences lacking exons responsible for transmembrane regions or parts of the catalytic domain.

FUCOSYLATED VARIANTS

The LN and LDN motifs of schistosomes are also prominently α3-fucosylated on GlcNAc, resulting in Le^X and LDNF, respectively. These trisaccharides function as both immunomodulators and antigens during infection. They are perhaps the best characterized of the C-type lectin ligands present in schistosomes and targeted by antibodies of many infected hosts, but their exact roles in infection have yet to be elucidated (Van Die et al., 2003; Van Vliet et al., 2005; Van Liempt et al., 2006; Meevissen et al., 2012a; Van Diepen et al., 2012a; Luyai et al., 2014; reviewed by Prasanphanich et al., 2013).

Both Le^X and LDNF have been documented on glycoproteins and glycolipids of all three major schistosome species (Nyame et al., 1998, 2000; Frank et al., 2012). Le^X is also a common feature of mammalian glycosylation, although it is often sulfated or sialylated (reviewed by Cummings, 2009). Its expression in schistosomes appears to be limited to the intramammalian stages and is especially prominent in the adult worm gut (Van Remoortere et al., 2000; Nyame et al., 2003; Peterson et al., 2009; Mandalasi et al., 2013). Le^X is also one of the major secreted schistosome antigens, with repeats of the antigen making up the polysaccharide portion of circulating cathodic antigen (CCA) found in serum and urine (Van Dam et al., 1994). LDNF appears to be expressed by all stages of the parasite, most highly by eggs and the intramolluscan stages (Van Remoortere et al., 2000; Nyame et al., 2002, 2003; Frank et al., 2012). In contrast, expression of LDNF is highly restricted in mammals—in humans it has been identified in urokinase and glycodelin (Bergwerff et al., 1992; Dell et al., 1995).

Alpha2- and α3-linked multifucosylated glycans are major constituents of a diverse group of immunologically important LDN derived epitopes. These epitopes contain unique linkages including polyfucose elements Fucα1-2Fucα1-3-R and the Fucα1-3GalNAc-motif generating F-LDN, F-LDN-F, LDN-DF and DF-LDN-DF variants (Khoo et al., 1995, 1997a; Kantelhardt et al., 2002; Peterson et al., 2013). These structures are not documented in any other parasitic or mammalian host species and induce high antibody responses in humans and primates (Van Remoortere et al., 2001, 2003; Kantelhardt et al., 2002; Naus et al., 2003). In fact, F-LDN-F is believed to be the motif responsible for the serological cross-reactivity with *S. mansoni* glycoconjugates and keyhole limpet hemocyanin (KLH) of the mollusc *Megathura crenulata* (Grzych et al., 1987; Kantelhardt et al., 2002; Geyer et al., 2004, 2005; Robijn et al., 2005). Additionally, the chitobiose core (-GlcNAcβ1-4GlcNAcβ1-) in complex type N-glycans can contain α6-linked fucose and the non-mammalian α3-linked fucose (Khoo et al., 1997a; Peterson et al., 2013). Such core modifications, especially α3-fucosylation, account for the interspecies immunological cross-reactivity observed among plant, insect, and helminth glycoproteins (Van Die et al., 1999; Paschinger et al., 2004; Peterson et al., 2013).

Prior to 2013 the fucosyltransferase (FucT) multigene family in *S. mansoni* was essentially unknown and most of the predicted

genes had not been substantively characterized (Marques et al., 1998; Trottein et al., 2000; Paschinger et al., 2005b). GeneDB designated 22 genes as putative FucTs with various specificities (α3-, α6-, O-). Two genes are further annotated as functioning on the core (Smp 154410) or generating Lewis structures (Smp 193620), however this activity has not been verified (Berriman et al., 2009). Analysis of the protein products from those genes revealed the database was incomplete, and the genes were fragments of what is expected in a full length FucT protein. Some gene products were prematurely truncated or missing exons in the stem or catalytic domains (Joziasse, 1992; Fukuda et al., 1996; Lairson et al., 2008). Ascertaining this problem with the database, Peterson et al. (2013) published a comprehensive *in silico* study using RACE (Rapid Amplification of cDNA Ends) PCR to determine the full-length transcripts of the FucT genes from a *S. mansoni* cDNA library. Their study identified six α3-FucTs (four new enzymes, one pseudogene, one previously discovered), six α6-FucTs, and two protein O-FucTs. Interestingly, no α2-FucTs were identified. The FucTs identified contain conserved motifs as well as characteristic transmembrane domains, consistent with their putative roles as fucosyltransferases (Breton et al., 1998; De Vries et al., 2001; Peterson et al., 2013). This new data, when grouped with previous transcript level results, suggest a possible mechanism for differential expression of fucosylated glycans in schistosomes (Fitzpatrick et al., 2009; Protasio et al., 2012; Peterson et al., 2013).

POLYLACTOSAMINE AND POLY-LDN

S. mansoni, like mammals, generates extended poly-N-acetyllactosamine (Galβ1,4-GlcNAcβ1,3-Galβ1,4-GlcNAc; poly-LN) chains which can be further modified, most notably in the form of poly-Lewis X (poly-Le^X) (Srivatsan et al., 1992b). Poly-Le^X has been demonstrated on N-glycans as well as on the secreted O-linked (possibly core 1 and/or core 2-linked) CCA (Bergwerff et al., 1994; Van Dam et al., 1994). Unusually, *S. mansoni* is also able to form extended polymers of LacdiNAc (GalNAcβ1,4-GlcNAcβ1,3-GalNAcβ1,4-GlcNAc; poly-LDN) and fucosylated LacdiNAc (poly-LDNF) (Wuhrer et al., 2006a,b). This is the only naturally-occurring example of such a structure; however, cloning of *C. elegans* β1,4-GalNAcT and human α1,3-fucosyltransferase 9 into Chinese Hamster Ovary Lec8 cells resulted in poly-LDN and poly-LDNF on N-glycans (Kawar et al., 2005). A β1,3-N-Acetylglucosaminyltransferase (β3GnT) in human serum also demonstrates extension activity in chemo-enzymatic generation of both poly-Le^X and poly-LDN on synthetic acceptors (Yates and Watkins, 1983; Salo et al., 2002). These data indicate that the β3GnTs which normally generate poly-LN in mammals are likely able to perform the reaction with either β-linked Gal or GalNAc as an acceptor. This is hypothesized to be the case in schistosomes as well (Wuhrer et al., 2006a), although the regulatory factors that allow extension of LDN in schistosomes but not in mammals are unknown.

Mammalian β3GnTs are part of a family of structurally-related β1,3-glycosyltransferase genes, which includes both GlcNAc- and Gal-transferases (Togayachi and Narimatsu, 2012). The *Schistosoma* genomes contain several genes homologous to this family, some of which are annotated as β3GnTs and others as β3GalTs, which have the conserved Galactosyl-T domain as well as a transmembrane region (Berriman et al., 2009; Zhou et al., 2009; Protasio et al., 2012). The enzymatic activities of the eight known mammalian β3GnT genes have been well-characterized, and each appears to have preferred substrates, such as β3GnT2, which extends poly-LN on 2,6-branches of tri- and tetra-antennary N-glycans, and β3GnT3, which extends poly-LN on O-linked core 1 (Togayachi and Narimatsu, 2012). As most of the *Schistosoma* genes have a similar level of protein sequence similarity to several of the mammalian β3GnT and vice versa, they will need to be cloned and biochemically characterized in order to determine which are responsible for extension of poly-Le^X, poly-LDN(F) on N-glycans and poly-Le^X on O-linked CCA, for example. A better understanding of the genetic basis of these polymeric antigens would be helpful as they are thought to be important antigenic targets, immunomodulators and, in the case of CCA, a validated diagnostic antigen (Van Dam et al., 1996; Van Roon et al., 2004; Wuhrer et al., 2006a; Sousa-Figueiredo et al., 2013; Luyai et al., 2014; Prasanphanich et al., 2014).

XYLOSE

Core β1,2-xylose linked to the β-mannose of N-glycans was first identified in plants and has since been recognized as a common modification of plant N-glycans and an important epitope of plant glycoprotein allergens. β2-xylosylation was subsequently identified in molluscs and then in *S. mansoni* and *S. japonicum* egg glycoproteins as well as *S. mansoni* cercariae in mass spectrometry studies (Khoo et al., 1997a, 2001). Western blot experiments suggest that several nematode and helminth species carry core β1,2-xylose and that it is variably expressed on glycoproteins of all of the intramammalian life stages of *S. mansoni*, with highest expression in cercariae and eggs (Van Die et al., 1999; Faveeuw et al., 2003). Core α3-fucosylated/core β2-xylosylated egg glycoproteins are also drivers of the Th2-immune response in mice and targeted by IgG in *S. mansoni*-infected mice, humans and rhesus monkeys (Faveeuw et al., 2003; Luyai et al., 2014). However, it is not clear what role such glycoconjugates play in schistosome infection, how they are developmentally regulated, and if antibodies to β1,2-xylose contribute to protection.

There are two xylosyltransferases annotated in the *S. mansoni* genome and three in the *S. japonicum* genome (Berriman et al., 2009; Zhou et al., 2009). Sjp 0055390 (Zhou et al., 2009) shares the greatest protein sequence similarity with other worm, mollusk and plant sequences annotated as β1,2-xylosyltransferases, including the well-characterized β1,2-xylosyltransferase from *Arabidopsis thaliana* (AtXYLT). AtXYLT is a type-II transmembrane protein, similar to other Golgi glycosyltransferases, with a conserved domain of unknown function (DUF563) that also occurs in the *S. japonicum* protein. AtXYLT adds a xylose β-linked to the central mannose of the N-glycan core structure, possibly acting at several points after the addition of GlcNAcβ1,2 to the α1,3-Mannose at the non-reducing end during Golgi N-glycan processing (Strasser et al., 2000; Bencúr et al., 2005; Kajiura et al., 2012). Smp 125150 is a shorter sequence which is also annotated as a β1,2-xylosyltransferase (Berriman et al., 2009), but may be a partial sequence as it aligns well with the N-terminal domain of Sjp 0055390 and AtXYLT but ends before

the conserved DUF commonly associated with β1,2-XYLTs. Transcriptome analysis (RNA-Seq) of the *S. mansoni* genome suggested that Smp 125150 expression was high in cercariae and decreased through the schistosomula stages to undetectable levels in adult worms (Protasio et al., 2012), which is potentially in agreement with the β2-xylosylation data described above from mass spectrometry and Western blot studies. These two genes therefore represent likely candidates for the *Schistosoma* β1,2-xylosyltransferases, and their improved characterization would benefit the developmental and immunological understanding of these worms.

CONCLUSIONS

The identification of novel glycans synthesized by schistosomes and their unique functions as immunomodulators and recognition as antigens has raised awareness of their importance. The complementary elucidation of the genomes of *Schistosoma* species has now opened the way to linking the glycogenome to the glycome, which has important consequences for the future of research in this area. Knowledge of specific genes encoding key parasite enzymes important in glycan synthesis may lead to new drugs targeted to block glycan synthesis or metabolism in the parasite. Such a strategy has the potential to target the parasites directly and/or to modulate the host's immune response to the parasite, both of which could have therapeutic value. The availability of identified and functional genes for schistosome glycosyltransferases could lead to their use in semi-synthetic strategies to produce glycans that are very difficult to obtain from chemical synthesis. Using chemo-enzymatic approaches it may be possible to generate a wide-variety of schistosome-related glycans and glycan determinants that would be ideal for screening of immune responses to glycan antigens in human and animal (Luyai et al., 2014; Prasanphanich et al., 2014). Finally, knowledge of the schistosome genes could lead to their use in recombinant forms expressed in mammalian or insect cells to elaborate the schistosome glycome in a heterologous cells for use in immunization and functional studies (Prasanphanich et al., 2014).

ACKNOWLEDGMENT

The authors acknowledge funding to Richard D. Cummings from NIH NIAID (R01AI101982) and from NIH NIGMS (P41GM103694 and GM098791).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 May 2014; paper pending published: 20 June 2014; accepted: 15 July 2014; published online: 05 August 2014.

*Citation: Mickum ML, Prasanphanich NS, Heimburg-Molinaro J, Leon KE and Cummings RD (2014) Deciphering the glycogenome of schistosomes. Front. Genet. **5**:262. doi: 10.3389/fgene.2014.00262*

This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Genetics.

frontiers in GENETICS

PERSPECTIVE ARTICLE
published: 31 July 2014
doi: 10.3389/fgene.2014.00229

Exploring the function of protein kinases in schistosomes: perspectives from the laboratory and from comparative genomics

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Eukaryotic protein kinases are well conserved through evolution. The genome of *Schistosoma mansoni*, which causes intestinal schistosomiasis, encodes over 250 putative protein kinases with all of the main eukaryotic groups represented. However, unraveling functional roles for these kinases is a considerable endeavor, particularly as protein kinases regulate multiple and sometimes overlapping cell and tissue functions in organisms. In this article, elucidating protein kinase signal transduction and function in schistosomes is considered from the perspective of the state-of-the-art methodologies used and comparative organismal biology, with a focus on current advances and future directions. Using the free-living nematode *Caenorhabditis elegans* as a comparator we predict roles for various schistosome protein kinases in processes vital for host invasion and successful parasitism such as sensory behavior, growth and development. It is anticipated that the characterization of schistosome protein kinases in the context of parasite function will catalyze cutting edge research into host-parasite interactions and will reveal new targets for developing drug interventions against human schistosomiasis.

Keywords: *Schistosoma*, kinome, *Caenorhabditis elegans*, kinase function, cell signaling, schistosomiasis

PROTEIN KINASES AND SCHISTOSOMES—AN OVERVIEW

Protein kinases are pivotal regulators of cellular function. When activated, these signaling enzymes phosphorylate transcription factors and other intracellular proteins leading to alteration in gene expression and/or other cellular behavior/activities. Elucidating functional roles for protein kinases in humans and other organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* has sometimes been challenging, particularly because protein kinase pathways can drive multiple functions and influence one another through cross talk (e.g., Krishna and Narang, 2008), the nature of which is often dependent upon "input" signal(s). This presents a conundrum when trying to characterize the functions of protein kinases in organisms that have only relatively recently entered the protein kinase research arena. Schistosomes, which are human blood parasites, arguably fall into this category. The importance of schistosomes, particularly *Schistosoma mansoni*, *Schistosoma japonicum*, and *Schistosoma haematobium*, is highlighted by the fact that they are responsible for causing the neglected tropical disease human schistosomiasis in over 200 million people worldwide (Steinmann et al., 2006).

The life cycle of these schistosomes is complex (Walker, 2011). Paired adult male and female worms living in the blood vessels of the human definitive host produce eggs that are excreted and hatch in fresh water releasing a miracidium that infects an aquatic intermediate host snail. In the snail the miracidium develops into a mother and then daughter sporocysts that produce cercariae; when released into water these cercariae search out and penetrate the skin of the human host. Each cercaria then transforms into a schistosomule which subsequently enters the circulation and develops into juvenile and then adult worms that pair to stimulate full maturation and egg laying. Considerable morphological and physiological differences exist between these separate schistosome life-cycle stages (Walker, 2011) and thus protein kinase function may be different in each individual life stage, adding a further layer of complexity to role characterization. Also, each life stage will intercept different "input" signals from its environment (Walker, 2011). Such signals will include: light and host-derived surface molecules in the free-living swimming stages (cercariae and miracidia); signaling molecules (e.g., growth factors) in the blood of the endoparasitic stages of both the snail (post-miracidia and mother/daughter sporocyst) and human (schistosomule and adult worm) host; and differences in osmolarity and/or temperature during infection of/release from the host. Furthermore, an immortalized schistosome cell line does not exist meaning that cellular experiments need to be conducted with whole schistosomes, fractions, or lysates thereof. Nevertheless, while challenging, defining roles for kinases in this fascinating parasite will be rewarding, enabling questions concerning cellular regulation, development and homeostasis, particularly during key life-stage transitions to be answered. Such research is also important to develop strategies focusing on drug-mediated kinase modulation in the parasite for therapeutic intervention.

Schistosoma mansoni, *S. japonicum*, and *S. haematobium* genome and transcriptome projects [e.g., (Hu et al., 2003; Verjovski-Almeida et al., 2003; Berriman et al., 2009;

The *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium, 2009; Protasio et al., 2012; Young et al., 2012)] have provided crucial sequence and expression data to support research into schistosome signaling, and 252 putative protein kinase genes have been found in *S. mansoni* (Andrade et al., 2011). However, the number of protein kinases encoded by these genes remains unknown because of the possibility of alternative splicing. This phenomenon, which increases proteome complexity, likely provides over 900 protein kinases from 445 protein kinase genes in humans, with 209 genes encoding a single kinase (Anamika et al., 2009). Alternative splicing results in potentially diverse functions because protein kinase splice variants can possess different domain architectures (Anamika et al., 2009).

Schistosomes possess putative kinases from all eight main eukaryotic protein kinase groups (Andrade et al., 2011). They also possess upstream receptors and endogenous signaling molecules (Osman et al., 2006; Khayath et al., 2007; Berriman et al., 2009; Oliveira et al., 2009; The *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium, 2009; Zamanian et al., 2011; Young et al., 2012). Importantly, in the context of host-parasite interactions, schistosomes have been shown to respond to human insulin (You et al., 2009), transforming growth factor (TGF)-β1 (Osman et al., 2006), and tumor necrosis factor (TNF)-α (Oliveira et al., 2009), demonstrating that they can bind host signaling molecules and transduce input signals through intact pathways. Here, perspectives on unraveling kinase function in schistosomes are provided that stem from techniques in cell biology developed for other organisms and from comparative genomics using *C. elegans* as a model.

SCHISTOSOME FUNCTIONAL KINOMICS—PERSPECTIVES FROM THE LABORATORY

STUDYING ACTIVATED PROTEIN KINASES IN SCHISTOSOMES

In our laboratory we have employed "smart" phospho-specific antibodies (Bonetta, 2005) to detect functionally activated protein kinases in life stages of *S. mansoni*. These antibodies, first produced by Cell Signaling Technology (CST; www.cellsignal.com) detect key phosphorylation sites (Tyr, Thr, or Ser) within the kinase that are critical for function. Although such antibodies are generated against phosphorylated human peptide sequences (typically 11–13 amino acids around the phosphorylation site), a number of such sequences are well conserved in kinases of invertebrates allowing certain antibodies to be used after careful validation. For example, anti-phospho antibodies against the mitogen-activated protein kinases (MAPKs) have been used widely in *D. melanogaster* and *C. elegans* (e.g., Gabay et al., 1997; You et al., 2006; Zhuang et al., 2006), and in snails (Plows et al., 2004, 2005) including *B. glabrata*, host to *S. mansoni* (Zahoor et al., 2008). In our approach (Ressurreição et al., 2011a) we first align the predicted *Schistosoma* kinase protein sequence (from www.GeneDB.org) with the orthologous sequence for the respective human kinase and search for conserved site(s) containing the important phosphorylation motif(s). We next select appropriate antibodies (e.g., from CST) and screen them using western blotting with extracts from untreated live parasites, and live parasites treated with an activator for the kinase in question (e.g., anisomycin for p38 MAPK). Increased immunoreactivity of the kinase from the exposed parasite samples provides the first indication of antibody suitability and of functional conservation of the phosphorylated site in schistosomes. Antibodies are then further validated before they are used routinely. This involves experiments such as inhibition assays to block upstream activators of the kinase or block direct kinase activation (Ludtmann et al., 2009), and if possible immunoprecipitation of the phosphorylated kinase followed by kinase assay. In principle, by raising antibodies against phospho-peptides identical to schistosome protein kinase sequences it is possible to produce schistosome-specific anti-phospho antibodies; however, this requires significant investment without knowing whether the phospho site is functionally active. A major benefit of using anti-phospho antibodies is that it is possible to study protein kinase activation with small quantities of protein (e.g., from ∼750 schistosomules or one adult worm pair) using western blotting. While for some schistosome protein kinases activity has been determined using activity assays with conserved kinase substrates (Wiest et al., 1992; Swierczewski and Davies, 2009, 2010), the quantity of protein needed is usually considerably greater. A further benefit of phospho-specific antibodies is that they often work in immunohistochemistry allowing the exclusively activated kinase (rather than the just the protein) to be visualized within the intact parasite using fluorescence-based confocal laser scanning microscopy (**Figure 1**). For this we have coined the term "functional mapping" (De Saram et al., 2013) given that only the activated kinase is detected. This can be valuable to elucidate the apparent distribution of the activated kinase within the parasite enabling hypotheses concerning function to be formulated and subsequently tested (see below). To date we have employed anti-phospho antibodies in *S. mansoni* to explore the kinetics of protein kinase C (PKC) and p38 MAPK activation in miracidia during development to mother sporocysts (Ludtmann et al., 2009; Ressurreição et al., 2011b), to help demonstrate a role for p38 MAPK in miracidia motility (Ressurreição et al., 2011a), and to study protein kinase A (PKA) function in adult worms (De Saram et al., 2013). Our current research using anti-phospho antibodies is focusing further on these pathways and others [e.g., AKT and extracellular signal-regulated kinase (ERK)] in cercariae, schistosomules, and adult worms.

We anticipate that the above tools will also help enable certain protein kinase-mediated signaling pathways to be delineated. Putative pathway maps have been constructed incorporating some schistosome protein kinases including MAPK pathways and others (Wang et al., 2006; Dissous et al., 2007; Berriman et al., 2009; Beckmann et al., 2010b). These are largely predicted using *in silico* data for schistosome pathway components, comparative mechanisms in vertebrates and, in some cases, interaction data from schistosomes obtained using valuable yeast two/three-hybrid screening and immunoprecipitation experiments [e.g., for Src and syk tyrosine kinases and polo-like kinases (Plks) (Quack et al., 2009; Beckmann et al., 2010a,b; Long et al., 2012)]. However, experimental data delineating routes of pathway activation in schistosomes remain negligible and need to be expanded to develop functional pathway maps.

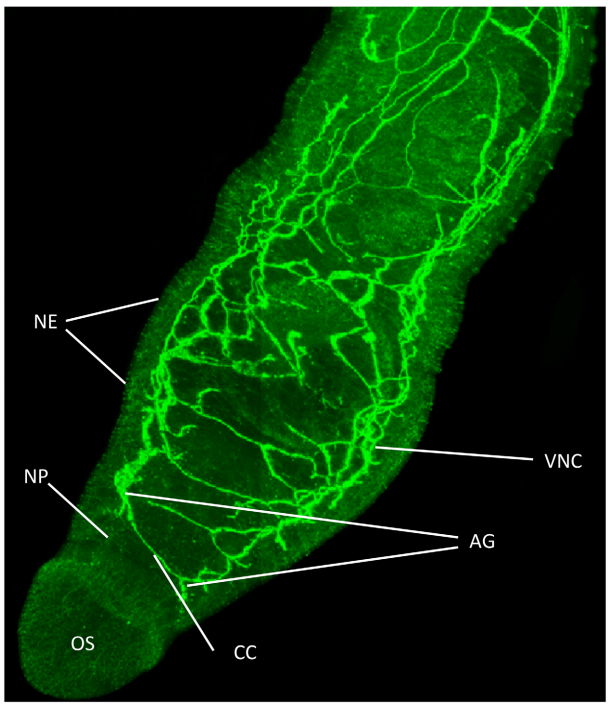

FIGURE 1 | Functional mapping of protein kinase A (PKA) in intact adult *S. mansoni*. Male worms were fixed immediately after perfusion and stained with anti-phospho antibodies (green) to localize activated PKA. Image shows z-axis projection from intact worm in maximum pixel brightness mode. Intense staining reveals PKA activation particularly associated with the nervous system including in the ventral nerve cords (VNC), connecting cerebral commissures (CC), anterior ganglia (AG), complex nerve plexus (NP) associated with the oral sucker (OS) and nerve endings (NE) at the tegument surface. Bar = 100 μm. Adapted from De Saram et al. (2013).

DECIPHERING PROTEIN KINASE FUNCTION IN INTACT SCHISTOSOMES

Current strategies for interfering with protein kinases to elucidate function directly within schistosomes focus largely on pharmacological inhibition and conventional RNA interference (RNAi) by double stranded RNA. Probably the most controversial aspect of using kinase inhibitors is their potential to affect "non-target" kinases, particularly at high concentrations (Anastassiadis et al., 2011). However, when functional experiments are performed on intact parasites inhibitor IC50's have little meaning as their values are normally derived from cell-free or single-cell assays. To help mitigate possible non-target effects, careful selection of kinase inhibitors is needed, particularly as they often have different specificities. Sometimes sites of protein-inhibitor interaction have been mapped as illustrated by SB203580 and its target p38 MAPK (Gum et al., 1998; Wang et al., 1998). Such knowledge is valuable as it enables one to ascertain whether the critical residues are conserved in the schistosome kinase, as was found for *S. japonicum* p38 MAPK during our recent work (Ressurreição et al., 2011a). Moreover, performing inhibition experiments with subsequent kinase activity screening (see above) further validates inhibitor use. Experiments with inhibitors/activators have supported roles for kinases in *S. mansoni* including in: (1) miracidia/sporocysts, in which p38 MAPK (Ressurreição et al., 2011b) and PKC (Ludtmann et al., 2009) inhibition restrict and accelerate miracidia-to-mother sporocyst development, respectively, and activation of p38 MAPK attenuates miracidial ciliary motion (Ressurreição et al., 2011a); (2) cercariae and adult worms, in which PKA inhibition kills the parasite (Swierczewski and Davies, 2009, 2010); and (3) adult worms, in which PKA activation stimulates neuromuscular movement (De Saram et al., 2013), insulin and venus kinase receptor inhibition restricts feeding, egg-laying, and results in death (Vanderstraete et al., 2013), and SmTK4 and Plk inhibition suppresses gametogenesis (Beckmann et al., 2010a; Long et al., 2010). Moreover, inhibition and transcriptomic analysis have recently been used to identify a co-operative role for Src kinase and TGFβ in eggshell formation (Buro et al., 2013). By coupling outcomes of pharmacological experiments with *in situ* functional mapping (De Saram et al., 2013)/*in-situ* hybridization (Long et al., 2012), further confidence in terms of identified role can be achieved. Thus, inhibitor-based assays have an important place in functional schistosome kinomic research when used appropriately, and are particularly useful in short-term experiments as RNAi-mediated knockdown of a protein can take several days.

Conventional RNAi has become an invaluable tool for studying protein function in various life stages of *Schistosoma* species (Kalinna and Brindley, 2007; Stefanić et al., 2010; Rinaldi et al., 2011). This approach has been used to silence a large number of schistosome proteins including leucine aminopeptidase, involved in hatching of miracidia (Rinaldi et al., 2009), tetraspanins 1 and 2 that regulate tegument integrity (Tran et al., 2010), cathepsin B, important to schistosome growth (Correnti et al., 2005), and aquaporin, involved in excretion of metabolic waste across the tegument (Faghiri et al., 2010). Challenges with RNAi in schistosomes, however, remain and are considered elsewhere (Stefanić et al., 2010; Dalzell et al., 2012). These include the transient nature of RNAi, variable knockdown between individual parasites, and "knock-down" rather than "knock-out" of gene function, all of which can complicate phenotype analysis. To-date, there are relatively few reports of schistosome protein kinases being targeted by RNAi. These include knockdown of PKA, SmTK4, fibroblast growth factor receptor, TGFβ receptor II and $Ca2^{+}$/calmodulin-dependent protein kinase II, found important for viability, gametogenesis, maintenance of neoblast-like cells, male-female reproductive development, and regulation of praziquantel induced calcium influx in adult worms, respectively (Osman et al., 2006; Swierczewski and Davies, 2009; Beckmann et al., 2010a; Collins et al., 2013; You et al., 2013). Importantly, the interconnected nature of kinase signaling is such that phenotypes caused by RNAi-mediated kinase depletion presumably reflect the aggregate biological consequence of disregulation of several pathways (Sopko and Andrews, 2008). Furthermore, phenotypes may also be masked by increased compensatory expression and subsequent activation of other isotypes and pathways in the face of suppression of any one isotype. Thus, although conventional RNAi is valuable for schistosome kinomics research, interpreting phenotype outcomes can be more challenging than for single gene/single function proteins. Nevertheless, in principle, as proposed for mammals (Moffat and Sabatini, 2006), it should

be possible to perform high-throughput RNAi-based screening to delineate schistosome signaling pathways, using downstream phosphorylation events as "readouts" for depletion of "upstream" components such as kinases. Although an immortalized schistosome cell line might appear essential for such experiments, we consider progress could also be achieved using primary cell cultures derived from mechanically or enzymatically dissociated schistosome tissues, and possibly even totipotent stem cells recently identified in this parasite (Collins et al., 2013; Wang et al., 2013).

SCHISTOSOME FUNCTIONAL KINOMICS—PERSPECTIVES AND PREDICTIONS FROM COMPARATIVE GENOMICS

Given that protein kinases have been conserved through evolution, valuable insights into their possible functions in schistosomes can also be gleaned by considering roles for orthologous kinases in organisms in which functional genomics is more advanced. Here, selected *S. mansoni* protein kinases displaying orthology to protein kinases of the well-characterized *C. elegans* have been chosen (**Table 1**) to illustrate how comparative genomics can help build hypotheses for testing protein kinase function in schistosomes.

Caenorhabditis elegans dauer larvae exhibit distinct morphological and behavioral characteristics in response to environmental duress. AKT-1 and AKT-2, components of the insulin-like pathway, phosphorylate and inhibit the FoxO transcription factor DAF-16 (Paradis and Ruvkun, 1998; Hertweck et al., 2004) regulating dauer formation and lifespan (Hu, 2007), such that DAF-16/FoxO activation in the intestine affects lifespan whereas neuronal activity affects dauer formation (Libina et al., 2003). The orthologous *S. mansoni* Akt gene (Smp_073930) is particularly expressed in the schistosomules and adult worms (**Table 1**); it is therefore possible to predict that Akt may regulate lifespan in *S. mansoni*. Interestingly, Smp_172240 is orthologous for a c-Jun N-terminal kinase (JNK-1) in *C. elegans*, which acts together with the insulin-like pathway and converges with DAF-16 to further promote lifespan regulation. In fact, *C. elegans* Jnk-1 mutants have reduced lifespan and increased tolerance to heat stress. JNK-1 modulates translocation of DAF-16 to the nucleus where it promotes expression of specific genes to combat environmental stress (Oh et al., 2005). Compared to cercariae, JNK-1 is more highly expressed in 3 h schistosomules (**Table 1**), which coincides with the considerable environmental change experienced by schistosomes upon entering the warm-blooded definitive host. The presence of *Schistosoma* insulin-like and JNK pathway components, vital to lifespan regulation in *C. elegans*, warrants investigations to decipher whether AKT and JNK regulate growth and development in schistosomes and whether these proteins co-operate in a similar manner. Thus, during our on-going investigations into AKT signaling in schistosomes we shall use the *C. elegans* knowledge base to build and test hypotheses concerning AKT function in different *S. mansoni* life stages.

Smp_065290, orthologous with CAM Kinase-1 in *C. elegans* and responsible for thermosensory behavior (**Table 1**), has raised expression in cercariae compared to 3 h schistosomules, consistent with cercariae being stimulated by a rising temperature gradients (Cohen et al., 1980). Although *C. elegans* CAMK-1 mutants display thermosensory defects, the mechanisms by which CAMK-1 operates remain to be characterized fully (Satterlee et al., 2004). Disruption of this gene in schistosomes would be interesting to revel whether it mediates host searching/cercarial penetration, possibly together with JNK-1 (orthologous to Smp_172240) that might play a role in thermosensation given its importance to thermotolerence in *C. elegans* (**Table 1**).

Schistosoma mansoni novel PKCε (Smp_131700; **Table 1**) is orthologous to *C. elegans* novel PKC-1 that plays a role in salt attraction (Adachi et al., 2010). Expression analysis (**Table 1**) suggests this gene is important in cercariae and early schistosomules. Increasing salinity promotes transformation of cercariae to schistosomules (Samueleson and Stein, 1989), thus, Smp_131700 provides an interesting target for phenotype disruption studies focusing on successful host infection by schistosomes. In *C. elegans*, PKC-1 is involved with ASE-right (ASER) neurons, which sense the Cl ion of NaCl (Adachi et al., 2010). ASER also express the guanylate cyclase receptor, gcy-22, essential in Cl ion sensing, and *C. elegans* gcy-22 mutants show impaired ability of ASER in response to varying salt concentrations (Ortiz et al., 2006). Interestingly, PKC-1 has other roles in *C. elegans* including nose touch stimulation and locomotion with evidence suggesting it acts through the conserved ERK pathway (Hyde et al., 2011). It is thus tempting to predict a further role for *S. mansoni* PKCε in mechanosensation during contact of cercariae to host skin and in schistosome locomotion. Future immunolocalization studies may reveal PKCε in the nervous system of *S. mansoni* as in *C. elegans*.

Other examples illustrated in **Table 1** include MAPK15, MEK1, casein kinase 1 (CK1), and vaccinia-related kinase (VRK), disruption of which in *C. elegans* is lethal to larvae, embryos, or both, and a G-protein coupled receptor kinase (GRK) that plays a role in chemotaxis, the hyperosmotic response and olfaction. The death-associated protein kinase (DAPK), expressed at high levels in all stages except 24 h schistosomules, plays an important role in epidermal and cuticle integrity and maintenance in *C. elegans* (**Table 1**). Given the importance of the schistosome tegument to host immune evasion and parasite survival (Van Hellemond et al., 2006), and the potential for similar effects in schistosomes, studies focusing on this kinase are warranted.

SCHISTOSOME FUNCTIONAL KINOMICS—FUTURE PERSPECTIVES

By integrating various approaches such as those detailed above and benefiting from emerging tools like schistosome transgenesis (Mann et al., 2011), it should be possible to unravel the complexity of protein kinase signaling in schistosomes. Certain protein kinases in schistosomes may ultimately be found to have roles comparable to those in other organisms including *C. elegans*, which can currently be used to generate functional hypotheses to test [see also further lethality predictions (Andrade et al., 2011)]. However, it is worth noting that common functionality may differ particularly as different organisms possess different complements of protein kinases and downstream target proteins, and because tissue expression may differ. To illustrate differences, *in silico* reconstruction of the *S. mansoni* and S. *japonicum*

Table 1 | Selected examples of orthologous protein kinases in *S. mansoni* and *C. elegans* with functional annotations shown for *C. elegans*.

S. mansoni Gene	Kinase type	Group	Family	Subfamily	Target ortholog(s) in *C. elegans*	Function of orthologous kinase in *C. elegans*. Notes curated from Uniprot (www.Uniprot.org), Wormbase (www.wormbase.org), and other referenced sources, including results of phenotype disruption experiments with *C. elegans*	References	*S. mansoni* QTE			
								Cerc	3h S	24h S	Adult
Smp_073930	Serine/Threonine Kinase	AGC	AKT		Q9XTG7 (akt2) and Q17941 (akt1)	Expressed in neurons, muscle cells of pharynx, rectal gland cells, and spermatheca. Simultaneous knockdown of akt-1 and akt-2 result in dauer formation/weak extension to life span. Role in immunity	Paradis and Ruvkun, 1998; Hertweck et al., 2004; Evans et al., 2008				
Smp_172240	Serine/Threonine Kinase	CMGC	MAPK	JNK	Q8WQG9 (jnk-1)	Expressed in neuronal cell bodies and processes. Analysis of mutant phenotype infers role in determination of adult lifespan and response to heat and oxidative stress. JNK kinase mutants exhibit locomotory defects	Kawasaki et al., 1999; Oh et al., 2005				
Smp_065290	Serine/Threonine Kinase	CAMK	CAMK1		Q9TXJ0 (CAM kinase 1)	Expressed in head and tail neurons and vuval muscles. Disruption results in changes in thermosensory behavior and temperature-dependent defects in AFD-specific gene expression	Eto et al., 1999; Kimura et al., 2002; Satterlee et al., 2004				
Smp_210820	Serine/Threonine Kinase	AGC	GRK	Beta ARK	Q09639 (grk2)	Broadly expressed in adult worm nervous system. Analysis of mutant phenotype infers role in chemotaxis, hyperosmotic response and olfaction	Fukuto et al., 2004				
Smp_131700	Serine/Threonine Kinase	AGC	PKC	Novel	P34885 (protein kinase C-like 1B)	Expressed in neurons that sense and respond to environmental signals. Disruption results in: attenuated response to nose touch stimulation, defects in salt attraction, disrupted chemotaxis, reduced dauer formation, reduced protection from hemiasterlin toxicity, reduced neuropeptide secretion	Okochi et al., 2005; Sieburth et al., 2007; Adachi et al., 2010; Zubovych et al., 2010; Hyde et al., 2011				
Smp_181490	Serine/Threonine Kinase	CAMK	DAPK	DAPK	O44997 (dapk-1)	Expressed in epidermis, muscles and neurons. Disruption reveals that beginning at L3, animals display progressive defects in morphology of the epidermis and cuticle, particularly the nose, tail, vulva, and the dorsal midline in the region of the posterior pharyngeal bulb. Thickened cuticle (5–10 times) at the expense of underlying epidermis. Up-regulates innate immune responses to damage. Decreases starvation-induced autophagy	Kang et al., 2007; Tong et al., 2009				

(Continued)

Table 1 | Continued

S. mansoni Gene	Kinase type	Group	Family	Subfamily	Target ortholog(s) in *C. elegans*	Function of orthologous kinase in *C. elegans*. Notes curated from Uniprot (www.Uniprot.org), Wormbase (www.wormbase.org), and other referenced sources, including results of phenotype disruption experiments with *C. elegans*	References	*S. mansoni* QTE			
								Cerc	3h S	24 h S	Adult
Smp_180400	Serine/Threonine Kinase	CK1	CK1	CK1D	Q8WQ99 (csnk-1)	Disruption results in 'dumpy' and 'small' phenotypes, egg variability, embryo and larval lethality, locomotory irregularity, paralysis and reduced brood size	Fraser et al., 2000; Simmer et al., 2003; Lehner et al., 2006				
Smp_141380	Serine/Threonine Kinase	CK1	VRK		Q19848 (vrk-1)	Expressed in germ cells, ventral nerve cord and vulval cells. Embryonically lethal. Analysis of mutant phenotype revealed VRK-1 essential for formation of the vulva, uterus and uterine seam cells and in development and maintenance of the somatic gonad and thus the germ line. Adults are sterile. Depletion leads to mitotic defects, including impaired nuclear envelope formation and baf-1 delocalization	Gorjánácz et al., 2007; Klerkx et al., 2009				
Smp_134260	Serine/Threonine Kinase	CMGC	MAPK	ERK7/ MAPK15	Q11179	Disruption results in larval arrest and is lethal to embryos	Rual et al., 2004				
Smp_026510	Serine/Threonine Kinase	STE	STE7	MEK1	Q10664 (mek-2)	Gene disruption reveals multiple phenotypes including: germ cell abnormalities, larval arrest, lethality, sterility, and developmental abnormality	e.g., (Sönnichsen et al., 2005; Ceron et al., 2007; Green et al., 2011)				

Relative quantitative transcript expression (QTE) data for cercariae (Cerc), 3 and 24 h schistosomules (3 and 24 h S), and adult worms were extracted from GeneDB [www.Genedb.org (Protasio et al., 2012)]; representing 0–0.2, 0.2–0.4, 0.4–0.6, 0.6–0.8, and 0.8–1.0 relative expression, respectively. Smp protein identifiers were queried in PhylomeDB (www.phylomedb.org) to obtain orthologous identifiers for C. elegans proteins, and protein sequence compared using BLAST (http://blast.ncbi.nlm.nih.gov). Curated data on Uniprot (www.Uniprot.org) and Wormbase (www.wormbase.org) were used for phenotype annotation.

MAPK pathways (Wang et al., 2006; Berriman et al., 2009) shows that while many of the *Schistosoma* cytosolic signaling mechanisms are intact when compared to those of humans, the number of orthologous downstream transcription factors appear fewer, with members such as CREB, p53, c-Myc, and c-Jun strikingly absent. Thus, while hypothetical frameworks for kinase signaling in schistosomes may be built, they do need to be tested.

Schistosome protein kinase research is arguably in its infancy and many questions remain concerning the functional biology of these important enzymes in this parasite. To help address these issues, and to stimulate research on schistosome protein kinases, it is proposed here that key areas for on-going fundamental schistosome kinomic research should include:

(i) Identification of upstream activators and downstream targets of schistosome protein kinases to enable functional pathway characterization. To include consideration of "input" signals such as host growth factors;
(ii) Functional elucidation of protein kinases beyond the commonly used "gross" phenotypes (e.g., dead/alive, moving/not moving) often evaluated;
(iii) Consideration of all life stages to fully appreciate the complexity of protein kinase signaling in this parasite. This is important because outcomes from one life-stage may offer novel insights into cellular regulation in another life-stage.

By expanding research in the above areas, it should be ultimately possible to integrate fundamental research outcomes and develop a systems level understanding of protein kinase function in schistosomes. In doing so, opportunities will emerge along the way to consider individual kinases as targets for drug-mediated chemotherapy for schistosomiasis. This may include making use of drugs that are available/being considered for therapy of other diseases such as cancer [e.g., (Ali et al., 2009)], modification of such drugs, or development of new multi-species drugs that specifically target *Schistosoma* protein kinases.

ACKNOWLEDGMENTS

The authors would particularly like to acknowledge Paulu de Saram (for **Figure 1**) and Marthe Ludtmann for their work on schistosome protein kinases at Kingston University, and David Rollinson and Aidan Emery both long-term collaborators at the Natural History Museum, London for their insightful discussions.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 February 2014; accepted: 30 June 2014; published online: 31 July 2014.
*Citation: Walker AJ, Ressurreição M and Rothermel R (2014) Exploring the function of protein kinases in schistosomes: perspectives from the laboratory and from comparative genomics. Front. Genet. **5**:229. doi: 10.3389/fgene.2014.00229*
This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Genetics.

frontiers in
GENETICS

REVIEW ARTICLE
published: 21 July 2014
doi: 10.3389/fgene.2014.00230

Schistosomes and snails: a molecular encounter

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Biomphalaria glabrata snails play an integral role in the transmission of *Schistosoma mansoni*, the causative agent for human schistosomiasis in the Western hemisphere. For the past two decades, tremendous advances have been made in research aimed at elucidating the molecular basis of the snail/parasite interaction. The growing concern that there is no vaccine to prevent schistosomiasis and only one effective drug in existence provides the impetus to develop new control strategies based on eliminating schistosomes at the snail-stage of the life cycle. To elucidate why a given snail is not always compatible to each and every schistosome it encounters, *B. glabrata* that are either resistant or susceptible to a given strain of *S. mansoni* have been employed to track molecular mechanisms governing the snail/schistosome relationship. With such snails, genetic markers for resistance and susceptibility were identified. Additionally, differential gene expression studies have led to the identification of genes that underlie these phenotypes. Lately, the role of schistosomes in mediating non-random relocation of gene loci has been identified for the first time, making *B. glabrata* a model organism where chromatin regulation by changes in nuclear architecture, known as spatial epigenetics, orchestrated by a major human parasite can now be investigated. This review will highlight the progress that has been made in using molecular approaches to describe snail/schistosome compatibility issues. Uncovering the signaling networks triggered by schistosomes that provide the impulse to turn genes on and off in the snail host, thereby controlling the outcome of infection, could also yield new insights into anti-parasite mechanism(s) that operate in the human host as well.

Keywords: intermediate snail host, *B. glabrata*, *S. mansoni*, resistance, susceptibility, compatibility, gene-expression, gene loci re-localization

INTRODUCTION

The neglected tropical disease (NTD), schistosomiasis, ranks second only to malaria as one of the most persistent debilitating diseases in impoverished areas of the tropics and sub-tropics, mainly in sub-Saharan Africa (Jenkins-Holick and Kaul, 2013). Freshwater snails are the obligate intermediate hosts for the transmission of the causative schistosome parasite. Using a combination of mass drug administration (MDA), mainly in school-aged children, and molluscicides to eliminate the snail from fresh water bodies, such as lakes, rivers, and their tributaries, low prevalence of schistosomiasis has now been achieved in several endemic countries (Tchuem Tchuente et al., 2013; Olveda et al., 2014). Despite these control efforts, however, reduction of schistosomiasis for the long term remains elusive, especially in poorer countries where access to clean water and sanitation remains challenging. Increasingly, strong advocacy by organizations, such as the schistosomiasis control initiative (SCI) and schistosomiasis consortium organization research effort (SCORE) have called for a renewed effort to better detect the parasite in snail populations where low prevalence of schistosomiasis has been accomplished (Fenwick et al., 2009a,b; Garba et al., 2009; Musuva et al., 2014). It is believed that by using an integrated control approach with aggressive surveillance complete global elimination of schistosomiasis might even become possible (Rollinson et al., 2013).

To make this goal a reality, however, novel intervention tools targeting the intermediate snail host are needed, especially since only one drug, praziquantel, effectively treats schistosomiasis and no vaccine is available (Hotez et al., 2010; Chai, 2013). Left untreated, schistosome adult worms can survive for several years (between 5 and 30 has been recorded). Eggs from mating not passed with human excreta but remaining trapped in tissues are the main cause of the pathology associated with schistosomiasis. This depends on the infecting parasite species, for example *Schistosoma haematobium*, can in time cause bladder cancer expedited from eggs sitting in tissues of this organ. Most notably, chronic *S. haematobium* infection can also cause female genital schitosomiasis (FGS), a condition that results in infertility and predisposes this section of society to HIV infection (Kjetland et al., 2010; Downs et al., 2013; Owusu-Bempah et al., 2013; Sahu et al., 2013).

Given the current interest in eliminating schistosomiasis, frequent use of molluscicides can have a negative impact on the environment as well as damage delicate ecosystems. Therefore, as was suggested as early as in the 1950's, a form of biological control

can be adopted by using incompatible snails to replace resident susceptible ones in endemic foci (Ruiz-Tiben et al., 1969; Ferguson and Ruiz-Tiben, 1971; Jobin and Laracuente, 1979). Indeed, in a proof of concept study conducted in the Caribbean Island of St Lucia, the snail *Biomphalaria straminae* a secondary host of the parasite *S. mansoni*, not its compatible snail *B. glabrata*, was utilized to eliminate schistosomiasis in the part of the island where this form of control was adopted (Pointier, 1993). In a more recent Brazilian study, the introduction of parasite resistant strains of *B. tenagophila* into an endemic site was found to reduce transmission as cross hybridization between resident susceptible and introduced snails increased over time (De Almeida Marques et al., 2014). This alternative intervention method, focusing on reducing schistosomiasis by blocking the snail stage of the parasite life cycle although attractive, will require a better understanding of the molecular basis of the snail and parasite interaction. This review will highlight recent advances that have been made toward unraveling molecular mechanisms that underlie parasite development in the snail host, particularly in the role of spatial epigenetics in the outcome of schistosome infection in the snail host.

GENETICS OF THE SNAIL HOST AND HOW THE PARASITE SHAPES THE OUTCOME OF INFECTION

Over the past two decades a major shift occurred in studies investigating snail/schistosome compatibility issues, from a comparative immunological point of view to one with a more molecular focus. Facilitating these studies was the existence of resources enabling investigators to identify genes involved in the snail's behavior toward the parasite. These include the *B. glabrata* embryonic cell line (Bge), and snail stocks that have been bred to display either resistance or susceptibility to *S. mansoni*.

Susceptibility of the snail host to the parasite has a genetic basis with genes of both the snail and parasite affecting the outcome (Spada et al., 2002; Theron and Coustau, 2005; Oliveira et al., 2008). For instance, resistance to infection in adult snail stocks, such as BS-90, 13-16-R1, and 10-R2 is a single dominant gene trait that is heritable by simple Mendelian genetics (Knight et al., 1991; Spada et al., 2002). Resistance in juvenile snails, however, is a complex trait that is controlled by several genes, five or six, each with multiple alleles (Ittiprasert et al., 2010).

Susceptibility to *S. mansoni* in *B. glabrata* snail stocks, such as M-line and NMRI is also a complex trait either as adults or juveniles. In some cases, snails can display susceptibility as juveniles, become resistant as adults, and revert to the susceptibility phenotype once they age (Knight et al., 1991; Richards et al., 1992). All these different outcomes of the parasite/snail encounter points to a relationship that is more variable and unpredictable in the snail than in the human host. Indeed, while a given parasite strain can only develop within a compatible snail, no such discrimination occurs in the human host. For this reason, it is highly plausible that discovery, in the snail host, of molecular pathways that either prevent or facilitate parasite development, if evolutionarily conserved, will help in designing tools that can be utilized for targeted control of schistosomiasis in both the snail vector and the human host.

GENE DISCOVERY HAS BEEN ACCOMPLISHED BY EXAMINING DIFFERENTIAL GENE EXPRESSION IN RESISTANT, AND SUSCEPTIBLE SNAILS

To determine the effect of parasite infection on *B. glabrata* gene expression, snail stocks that are either resistant (BS-90) or susceptible (NMRI, and M-line), exposed at various time points to *S. mansoni*, were examined by a variety of approaches to assess the degree of parasite-mediated modulation of the snail transcriptional profile. The variety of methods utilized to examine differential gene expression between these snails, included generating expressed sequence tags (ESTs) from either intact snails or specific tissues (hemocytes, ovotestis, mantle, hepatopancreas, and albumen gland), differential display reverse transcription-polymerase chain reaction (DD-RT-PCR), microarray analysis, and RNA-sequencing (RNA-seq; Knight et al., 2000; Raghavan et al., 2003; Mitta et al., 2005; Lockyer et al., 2007, 2008; Ittiprasert et al., 2009, 2010). In addition, a suppression subtractive hybridization (SSH)-cDNA cloning strategy was employed to enrich for transcripts featuring prominently between resistant and susceptible snails during the early response phase to the parasite (Ittiprasert et al., 2010).

From these various profiling experiments, results showed that transcripts involved in the snail's innate defense system (IDS) were amongst those that were most significantly upregulated after early exposure to the parasite, especially in resistant snails. Thus in resistant snails, such as the BS-90 stock, compared to susceptible ones, e.g., the NMRI, and M-line snail stocks, immune-defense behavior of the snail/schistosome interplay, involving cellular, "macrophage-like" cells known as hemocytes, and the plasma, hemolymph, component, recognize the incoming parasite (larval miracidium) as non-self by an as yet unknown mechanism. This recognition results in the encapsulation of the parasite by hemocytes released into the open circulatory system from the snail's hemopoietic tissue, the amoebocyte producing organ (APO) located in the pericardiac and renal regions (Souza Sdos and Andrade, 2006, 2012). The nature of the excretory-secretory products (ESPs) released from the parasite that stimulate hemocyte production, recruitment, and attachment to the parasite surface, remain unknown. However, hemocyte genes that are up-regulated in response to exposure to schistosomes and their ESPs are being identified (Zahoor et al., 2009, 2014; Lockyer et al., 2012). Aided by plasma products, including lectins, hemocytes encapsulate the parasite within a few hours post-exposure, and by a cytotoxic mechanism(s) that include the production of reactive oxygen and nitrogen intermediates, kill the parasite (Hahn et al., 2001).

Hemocytes passively transferred from a resistant to susceptible snail have been shown to be capable of rendering these snails less susceptible (Vasquez and Sullivan, 2001). This active anti-schistosome defense reaction in resistant snails does not occur in those that are compatible/susceptible. In this case, the prevailing hypothesis is that the parasite is capable of suppressing the snail IDS, leading to the successful establishment and development of the early mother sporocyst stage (Yoshino and Bayne, 1983; Mattos et al., 2011). What mechanism(s) are involved in the manipulation of the snail's defense by the parasite has yet to be uncovered but from comprehensive transcriptomics, several lectins, including

highly diversified transcripts encoding the fibrinogen encoding proteins, collectively known by the acronym fibrinogen-related proteins (FREPs) are upregulated in both resistant and susceptible snails. Studies using experimental dsRNA to knock down the transcript for FREP 3 in resistant snails rendered them susceptible, indicating that FREPs plays a role in anti-trematode defense not coagulation (Zhang and Loker, 2004; Hanington et al., 2012). The unusual diversification of FREPs, the first to be discovered for a defense-related molecule in invertebrates, points to an IDS that is more complex than was previously envisioned. Snail lectins, including FREPs also bind to released parasite molecules, such as mucin (Roger et al., 2008a; Zhang et al., 2008; Hanington et al., 2010). This interaction between snail and parasite molecules that have co-evolved sufficiently to "match" has been proposed as underlying snail/schistosome compatibility (Massoud et al., 2004; Oliveira et al., 2008; Roger et al., 2008a,b; Mitta et al., 2012). Lately, evidence for specific-genotype immune priming has also been detected in the *B. glabrata*/schistosome interaction (Portela et al., 2013). Thus far, however, interacting components known to trigger innate immunity in other invertebrates, specifically pathogen-associated molecular patterns (PAMPs), as well as host pattern recognition receptors (PRRs), including signaling PRRs, e.g., the membrane-bound Toll receptors remain to be characterized for the *B. glabrata/S. mansoni* relationship (Meurs et al., 2011; Paveley et al., 2011).

Considerable progress is being made on identifying shared molecular moieties in the parasite and snail host, and evidence is emerging that shows the existence of shared specific glycotypes (glycan epitopes) between the larval schistosome parasite and snail hemocytes (Yoshino et al., 2013). It is believed that the shared epitopes, also called molecular mimicry might be used to dampen immune recognition of the parasite thus evading immune detection.

STRESS INDUCTION BY SCHISTOSOMES IN THE SNAIL HOST CORRELATES WITH SUSCEPTIBILITY

From studies that compared the temporal regulation in gene expression between resistant and susceptible snails exposed to *S. mansoni*, it was detected that several stress related transcripts were up-regulated early and significantly in susceptible juvenile snails compared to their resistant counterparts (**Figures 1A,B**; Ittiprasert et al., 2010). For example, transcripts encoding heat shock protein (Hsp) 70, Hsp 90, and the reverse transcriptase (RT) domain of the *B. glabrata* non-LTR retro-transposable element, *nimbus*, were detected in susceptible NMRI snails at early time points (5 h) post-exposure compared to the resistant BS-90 snail. Furthermore, upregulation of the aforementioned stress related transcripts was observed in snails exposed to normal but not to irradiated miracidia (Ittiprasert et al., 2010).

GENOME ORGANISATION IN *B. glabrata* IS SIMILAR TO HIGHER ORGANISMS

In order to understand how the parasite is able to induce and even regulate gene expression – such as the heat shock genes, it is imperative to understand the functional genome biology and its organization within the host cell nuclei. Furthermore, to fully appreciate how the parasite is controlling gene expression the structures that are involved in genome organization and behavior also need to be elucidated (see **Figures 2A,B,F,G**).

When originally analyzing the organization of the snail genome within cell nuclei it was hypothesized that it might be more similar to simpler organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*. Interestingly, it was found that the genome organization within the snail was similar to the mammalian genome. This was discovered by the preparation of a single chromosome painting probe that when used in fluorescence *in situ* hybridization (FISH) delineated individual chromosome territories (**Figure 2C**; Knight et al., 2009), these resembled territories found in higher organisms such as human, mouse (Foster et al., 2005) and pigs (Foster et al., 2012) and was also found to be non-randomly located within cell nuclei. Large and small chromosome territories were also revealed (Odoemelam et al., 2009), although it is as yet not possible to reveal gene density correlation with chromosome location since sequencing of the genome remains unfinished. However, the karyotype of *B. glabrata* has now been completed and several chromosomes have now been named and identified by utilizing bacterial artificial chromosomes (BAC) probes generated by the genome sequencing effort (Arican-Goktas et al., unpublished data). From these data, it is clear that *B. glabrata* chromosomes are not as previously described, i.e., being monomeric since they display differences in size, centromere position, and banding, implying there are gene-rich and gene-poor chromosome regions as well as gene-rich and -poor chromosomes. This advance will be very important for the genome sequencing effort as it will allow genes to be located to a specific chromosome, facilitating assembly, annotation, and organization of the sequence data. The protocol development for mapping onto *B. glabrata* chromosomes by FISH has taken a decade to refine and these methods will be published with the manuscript describing the genome.

The genome has some role in organizing itself within nuclei – through chromatin modification, i.e., the histone code (**Figures 2D,E**), but it also requires many different proteins within nuclei to help position and tether it. A concerted effort has been made to study *B. glabrata* cell nuclei to determine how similar they are to other organisms. As mentioned above, the nucleus of *B. glabrata* appears to be very similar to mammalian cell nuclei (**Figure 2**), containing many nuclear structures that were revealed using indirect immunofluorescence with antibodies reacting with human antigens, such as nuclear lamins (**Figure 2A**), nucleoli (**Figure 2B**), nuclear bodies (**Figure 2F**), nuclear motors (**Figure 2G**), transcription factories and splicing speckles, making this mollusk an interesting model to analyze genome behavior in since it has aspects of both simpler and more complex organisms.

SCHISTOSOMES INFLUENCE REPOSITIONING OF GENE LOCI IN INTERPHASE NUCLEAR, CORRESPONDING WITH TRANSCRIPTION

The complex relationship between the schistosome parasite and a given compatible species of snail developed over many 1000s of years, and is a co-evolved relationship.

Using labeled BAC probes and FISH analysis, gene loci position has been mapped in *B. glabrata* and found as small foci, very similar to other organisms (**Figure 2H**; Odoemelam et al.,

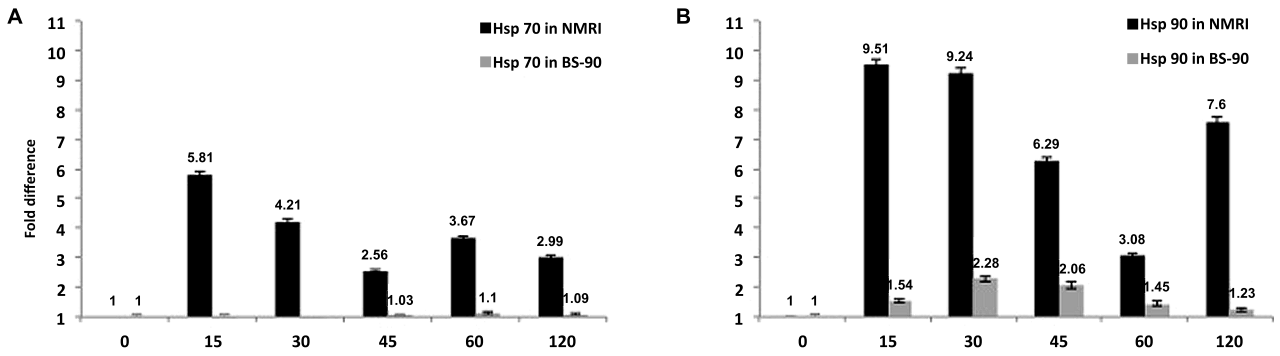

FIGURE 1 | Temporal modulation of the transcript encoding (A) Hsp 70 or (B) Hsp 90 in susceptible NMRI and resistant (BS–90) *B. glabrata* snail stocks examined by qPCR after exposure (0 – 120 min) to *S. mansoni* miracidia. Note that expression of the transcripts occurs only in the susceptible but not in the resistant snail (Ittiprasert et al., 2010; Ittiprasert and Knight, 2012).

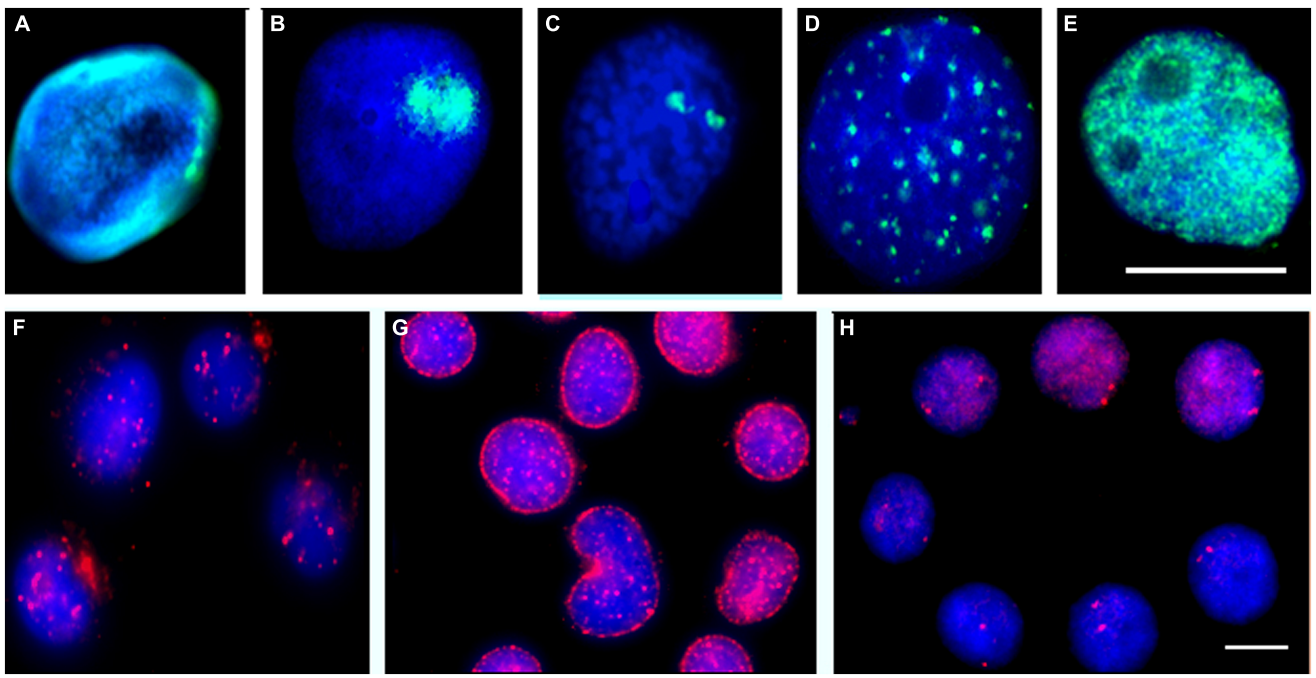

FIGURE 2 | *Biomphalaria glabrata* embryonic (Bge) cells grown *in vitro* or *ex vivo* cells extracted from *B. glabrata* snails stained with antibodies or subjected to FISH. Antibodies raised against other species were used to reveal similar nuclear structures within snails and Bge cells; these include the nuclear lamina (**A**, green), fibrillarin in the nucleolus (**B**, green), PML bodies (**F**, red) and nuclear myosin (**G**, red). Chromatin modification was revealed using antibodies made against *D. melanogaster* modification that have a broad species range; histon 4 trimethylated K27 (**D**, green) and histone 3 trimethyl K4 (**E**, green). Individual chromosome territories were delineated by allowing 5-bromo-2′-deoxyuridine to be incorporated into replicating DNA and then allowing cells to divide seven times, followed by indirect immunofluorescence with an anti-5-bromo-2′-deoxyuridine antibody (**C**, red). Individual gene loci were revealed by FISH using labeled BAC probes (**H**, red). The DNA of the cells was stained with a DNA intercalating dye 4′6-diamidino-2-phenylindole. Scale bar 10 μm (Drs. Arican-Goktas, 2013 and Odoemelams′, 2009, Ph.D. thesis, Brunel University, UK) (Odoemelam, 2009; Arican-Goktas, 2013).

2009). Using a bespoke computer analysis script (Croft, 1999), the positioning of these gene loci is found to be non-random with some genes being found at the nuclear periphery, some in the nuclear interior and some in an intermediate location as for other organisms (Szczerbal et al., 2009; Bourne et al., 2013). However, upon exposure to the parasite either *in vitro* with Bge cells (Knight et al., 2011) or *in vivo* in intact snails we found that specific genes change their nuclear location to a new non-random location correlating with their expression. For most genes attenuated parasite failed to elicit either a relocalization of gene loci, or change in expression. The *in vivo* studies have been most revealing since we were able to employ both susceptible and resistant snails. For gene loci corresponding to Hsp 70, we found overwhelming differences in behavior between the two snail lines. For example, in the susceptible NIMR snail, the gene loci for Hsp 70 moved to a new nuclear location before being expressed whereas no such

movement and no gene expression for Hsp 70 were observed in the resistant BS-90 snail, corroborating previously reported differential gene expression data for Hsp 70 between resistant and susceptible snails (Knight et al., 2011). Additionally, when attenuated parasite was used to infect these snails, neither gene movement nor expression of Hsp 70 was observed. From these data, we believe the movement and expression of Hsp 70 is controlled by the parasite infection and may be used to help promote its survival/development in the snail (Arican-Goktas et al., 2014) parasite elicit a gene movement to a new nuclear location for gene expression to occur? We postulate that this requires remodeling of chromatin at specific genome locations and active directed movement to an area of active transcription in the cell nuclei using nuclear motor proteins such as nuclear myosin 1b (**Figure 2G**).

CONCLUSION

In the 20 years since a molecular approach to decipher mechanisms of the snail host/ schistosome interaction was undertaken – a relationship fashioned over centuries to give each organism a survival advantage over the other, we have discussed in this review how important advances have indeed been made. For example, the long held belief that innate defense molecules of invertebrates, unlike those of vertebrates (e.g., antibody) lack the ability to diversify in their structure has been decisively proven incorrect by the level of diversification revealed in *B. glabrata* FREP transcripts. Likewise, we now also know that schistosomes have evolved a mechanism that is capable of manipulating the genome of a compatible snail host, thereby orchestrating changes in the snail's cell nuclei that alters gene expression in the parasite's favor. While these important groundbreaking advances have been made, however, more work remains to be done. For example, we still do not know how schistosomes induce relocalization of gene loci to nuclear compartments for gene expression or silencing. These questions notwithstanding, it is clear that with the entire sequence of the genome soon becoming available, *B. glabrata* should fast become a new model system whereby the effect of a complex pathogen on genome behavior can be examined, enabling us to discover novel pathways that can be interfered with to disable snail transmission of schistosomiasis.

ACKNOWLEDGMENTS

We thank the NIH – NIAID for the grant award (R01AI63480) that funded the work. Halime D. Arican-Goktas was partially funded by the Malacological Society of London. We also thank Drs. Victoria Mann and Jacob Hodgson for their helpful critique of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 April 2014; accepted: 30 June 2014; published online: 21 July 2014.
*Citation: Knight M, Arican-Goktas HD, Ittiprasert W, Odoemelam EC, Miller AN and Bridger JM (2014) Schistosomes and snails: a molecular encounter. Front. Genet. **5**:230. doi: 10.3389/fgene.2014.00230*
This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Genetics.

frontiers in
GENETICS

MINI REVIEW ARTICLE
published: 18 July 2014
doi: 10.3389/fgene.2014.00238

Receptor tyrosine kinases and schistosome reproduction: new targets for chemotherapy

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Schistosome parasites still represent a serious public health concern and a major economic problem in developing countries. Pathology of schistosomiasis is mainly due to massive egg production by these parasites and to inflammatory responses raised against the eggs which are trapped in host tissues. Tyrosine kinases (TKs) are key molecules that control cell differentiation and proliferation and they already represent important targets in cancer therapy. During recent years, it has been shown that receptor tyrosine kinases (RTK) signaling was active in reproductive organs and that it could regulate sexual maturation of schistosomes and egg production. This opens interesting perspectives for the control of transmission and pathogenesis of schistosomiasis based on new therapies targeting schistosome RTKs. This review relates the numerous data showing the major roles of kinase signaling in schistosome reproduction. It describes the conserved and particular features of schistosome RTKs, their implication in gametogenesis and reproduction processes and summarizes recent works indicating that RTKs and their signaling partners are interesting chemotherapeutical targets in new programs of control.

Keywords: schistosome, receptor tyrosine kinase, signaling, reproduction, chemotherapy

SCHISTOSOMES, KINASE SIGNALING, AND REPRODUCTION

Schistosomiasis, or bilharzia, is a parasitic disease with outstanding medical and economic importance, caused by trematodes of the *Schistosoma* genus (Chitsulo et al., 2004). *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum* are the three major infective species in humans. Whereas most of the trematodes are hermaphrodites, schistosomes have separate sexes, and the sexual maturation of female worms is dependent on constant pairing with males (Popiel and Basch, 1984; LoVerde and Chen, 1991; Kunz, 2001). Pairing induces mitosis and maintains differentiation of stem cell-like precursor cells in the ovary and vitellarium (Erasmus, 1973; Den Hollander and Erasmus, 1985; Kunz, 2001; Galanti et al., 2012). These processes are a prerequisite for the production of eggs, formed by one oocyte and 30–40 vitelline cells combined within the ootype to form viable eggs. The control of this major parasitic disease currently relies on mass treatment with a single drug, Praziquantel. This drug is efficient against the three human schistosome species, but its widespread use raises fears about resistance and motivates the search for alternative therapies (Doenhoff et al., 2008; Melman et al., 2009). During recent years, much effort has been made to understand the development of female reproductive organs with the aim to find strategies to reduce transmission and morbidity of schistosomiasis by preventing egg production (LoVerde et al., 2009; Beckmann et al., 2010a). Indeed, eggs are responsible for parasite transmission but also for pathogenesis in humans since they accumulate in host tissues, particularly in liver, and elicit granulomatous inflammatory reactions leading to periportal fibrosis, portal hypertension, and hepatosplenomegaly (Hoffmann et al., 2002).

Evidence has been obtained that TGF-β pathways play a major role in female reproductive development and egg embryogenesis. Essential components of TGF-β pathways (TbRI and II, R-Smad, Co-Smad, FKBP12) have been identified in schistosomes, and their expression in vitelline cells was demonstrated (Davies et al., 1998; Beall et al., 2000; Osman et al., 2001, 2004, 2006; Knobloch et al., 2004). The TGF-β pathway regulates the synthesis of SmGCP, a gynecophoral canal protein involved in promoting contact between males and females (Osman et al., 2006). The activin ligand of the receptor TbRII, SmInAct, is also crucial for successful embryogenesis in schistosome eggs (Freitas et al., 2007) and the TbRI serine/threonine kinase inhibitor (TRIKI) was shown to reduce vitelline cell mitotic activity and egg production in female worms (Knobloch et al., 2007). Tyrosine kinases (TKs) are also important for female gonad development and in this context, the Src-like cytosolic TK (CTK), SmTK3, seems to play a dominant role in proliferation of vitelline cells (Kapp et al., 2004). Treatment of parasites with the Src inhibitor Herbimycin A significantly blocked male-induced mitotic activity in paired females and interestingly, the combined treatment of paired schistosomes with TRIKI and Herbimycin A was more efficient to reduce mitosis and egg production, indicating possible cross-talk between Src and TGF-β pathways (Knobloch et al., 2007). Recently, transcriptome analyses of inhibitor-treated schistosomes provided further evidence for a cooperation between Src-kinase and TGF-β pathway in the control of mitosis and eggshell formation (Buro et al., 2013) and for an association of Abl-kinase activities with TGFβ signaling (Buro et al., 2014). Finally, the Syk kinase SmTK4 (Knobloch et al., 2002a,b) and the Src/Abl hybrid kinase SmTK6

(Beckmann et al., 2011) presumably act together with SmTK3 in a multi-kinase complex to transduce signals potentially induced by the activation of membrane receptors, such as integrins (Smβ-Int1) or receptor tyrosine kinases (RTKs) and which are important for gametogenesis in parasite gonads (Beckmann et al., 2011, 2012).

SCHISTOSOME RECEPTOR TYROSINE KINASES

Receptor tyrosine kinasess play essential roles in embryonic development and in various adult tissues and organs, in which they control fundamental processes, such as cell proliferation and differentiation, cell cycle and survival, migration, and metabolism (Ullrich and Schlessinger, 1990). RTKs form a superfamily of transmembrane proteins present in all metazoans, from sponges to humans (Suga et al., 2012) and they are composed of an extracellular ligand binding region formed by various subdomains, a single transmembrane domain, and an intracellular domain with intrinsic TK activity. Annotation of the *S. mansoni* genome (Berriman et al., 2009; Protasio et al., 2012) and analyses by combined computational approaches have shown that the tyrosine kinome of *S. mansoni* contains 15 RTKs including four members of the epidermal growth factor receptor (EGF-R) family, two of the IR (insulin receptor) family, two FGF-R (fibroblast growth factor receptor) members, one representative of Ephrin-R, ROR, and MuSK families, a homolog of CCK4, and one unknown receptor (Andrade et al., 2011; Avelar et al., 2011). Additionally, the schistosome genome encodes two Venus kinase receptors (VKRs), belonging to a novel family of RTKs originally discovered in *S. mansoni* (Vicogne et al., 2003; Dissous et al., 2014a,b).

Over the past few years, five RTKs of *S. mansoni* have been particularly well investigated. SER (*Schistosoma* EGF-R) was the first RTK described in *S. mansoni* (Shoemaker et al., 1992). It contains a conserved intracellular TK domain and an extracellular domain for binding of EGF ligands. When expressed in mammalian cells, SER can bind human EGF with the same affinity as human EGF-R (HER) and it activates the classical and conserved Ras/ERK signaling pathway. Human EGF was shown to induce SER autophosphorylation in *S. mansoni* adult worms and to increase protein and DNA synthesis as well as protein phosphorylation in schistosome larvae, indicating for the first time that host hormones were involved in regulating schistosome development (Vicogne et al., 2004). Such a potential dialog between host ligands and parasite receptors was further confirmed by the ability of human TGF-β to activate TbRI/II schistosome receptors (Beall and Pearce, 2001) but also by evidence that human insulin can bind to schistosome membrane RTKs to regulate metabolic and glucose uptake activities in the parasite (Khayath et al., 2007; Ahier et al., 2008; You et al., 2010). Two members of the IR family were identified in *S. mansoni* (SmIR1 and SmIR2; Khayath et al., 2007), then in *S. japonicum* (SjIR1 and SjIR2; You et al., 2010). Schistosome IR1 and IR2 display differences in the structural motifs essential for signaling and in their expression sites. Schistosome IR1 is expressed in muscles, intestinal epithelial cells and at the basal membrane of the tegument (Khayath et al., 2007), and they are colocalized with SGTP1 and SGTP4, the schistosome glucose transporters involved in glucose uptake (Skelly et al., 1994). Schistosome IR2 is massively expressed in parenchymal cells of adult schistosomes (Khayath et al., 2007; You et al., 2010), and SjIR2 was also localized in vitelline cells (You et al., 2010). A single IR is present in most invertebrate species which regulates both metabolism and growth, while two receptors IR and IGF-1R exist in vertebrates, which are specialized in metabolic and glucose uptake regulation, and in growth control, respectively (Kim and Accili, 2002). It was suggested that similarly SmIR1 and SjIR1 could be specialized in sugar uptake and SmIR2 and SjIR2 preferentially involved in growth of schistosomes.

Besides these conventional and conserved RTKs, which are able to respond to host growth factors (EGF, insulin) and to activate conserved signaling pathways, schistosomes were shown to express unconventional RTKs, named VKRs for Venus kinase receptors (Vicogne et al., 2003; Ahier et al., 2009). VKRs are composed of an extracellular Venus flytrap module (VFT), linked through a single transmembrane fragment to an intracellular TK domain similar to that of IRs (Vicogne et al., 2003). VFTs are the extracellular domains of many G-protein coupled receptors of class C, and they are composed of two lobes that close upon the binding of small ligands (amino-acids, ions) similarly to the leaves of the Venus flytrap carnivorous plant, *Dionaea muscipula*, when it catches its prey (Pin et al., 2003). VKRs are found in a large variety of invertebrates from cnidarians to echinoderms (Ahier et al., 2009; Vanderstraete et al., 2013a), and are highly expressed in larval stages and in gonads, suggesting a role of these proteins in embryonic and larval development as well as in reproduction (Ahier et al., 2009; Vanderstraete et al., 2014). *Vkr* genes are found as single copies in most species but in platyhelminths two different *vkr* copies are present (Gouignard et al., 2012; Vanderstraete et al., 2013a, 2014). Up to now, two VKRs have been found in *S. mansoni* as well as in *S. haematobium* (Young et al., 2012). SmVKR1 and SmVKR2 of *S. mansoni* have been extensively studied. They were shown to be activated by L-arginine and calcium ions, respectively (Gouignard et al., 2012) and, as many other RTKs, VKRs dimerize to induce intracellular pathways involved in protein synthesis and cellular growth, like the MAPK and PI3K/Akt/S6K pathways (Dissous et al., 2014a; Vanderstraete et al., 2014).

Among the growth factor receptor panel, EGFR and IR/IGFR have been shown to play essential roles in mammals (Schneider and Wolf, 2008; Sirotkin, 2011) as well as in insects (Graf et al., 1997; Brown et al., 2008; Parrott et al., 2012) for the control of ovarian functions and reproductive processes. Additionally, the discovery that VKRs were abundantly transcribed in the gonads of many invertebrate species (Ahier et al., 2009), indicated that VKR signaling could also participate in reproductive activities. Further analyses have confirmed that all these RTKs were potentially involved in reproduction processes in schistosomes (**Figure 1A**).

FUNCTIONS OF SCHISTOSOME RTKs IN REPRODUCTION

Besides the importance of the receptor Ser/Thr (S/T) kinases SmTbRI/II and of TGF-β-dependent pathways in schistosome reproduction processes (LoVerde et al., 2007, 2009), TK signaling is also predominant for schistosome gonad development (Beckmann et al., 2010a,b), and a possible cooperation between STK and TK pathways has been proposed for the control of mitosis

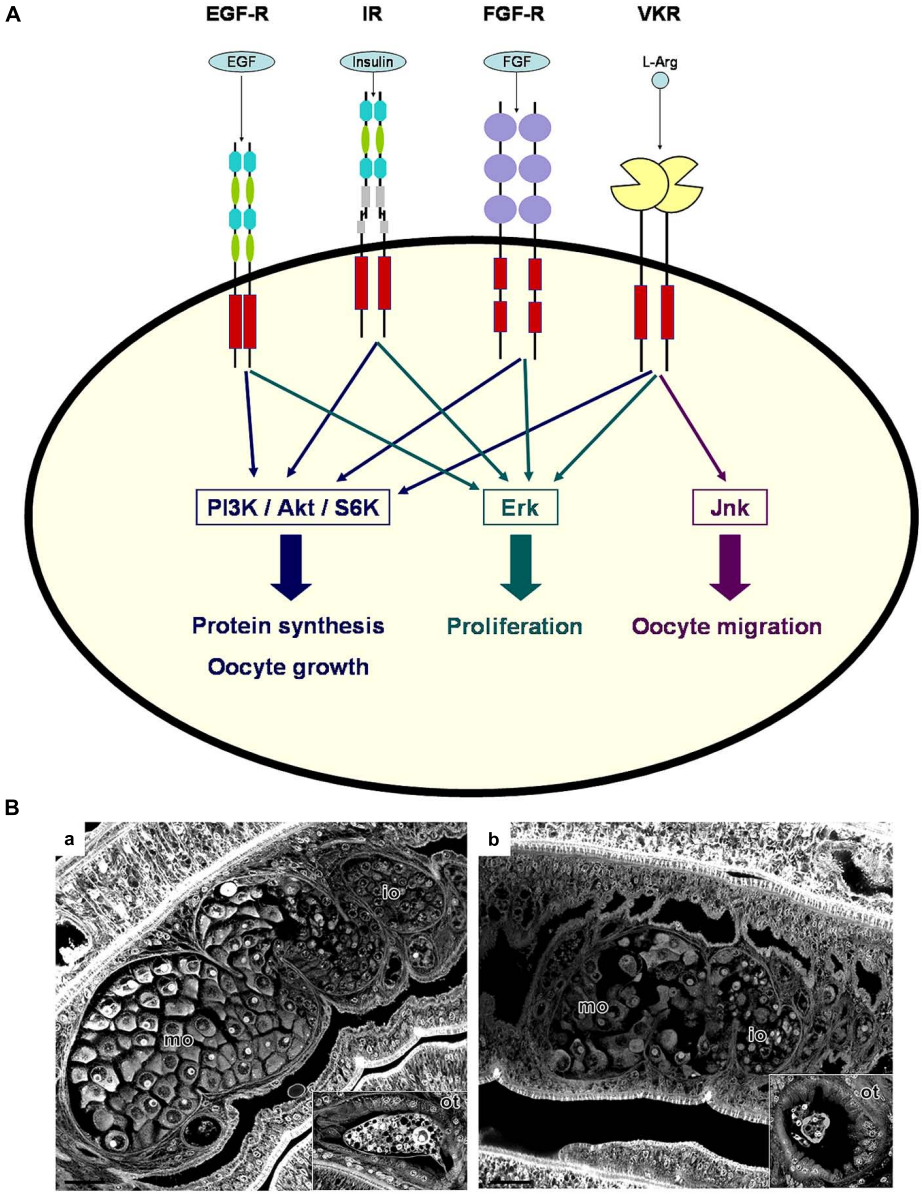

FIGURE 1 | Receptor tyrosine kinase (RTK) signaling in schistosome gonads. (A) Following activation by their respective ligands, schistosome RTKs (EGF-R, IR, FGF-R, and VKR) expressed in gonads can induce protein synthesis, cell growth and proliferation by activating the PI3K/Akt/S6K and Erk MAPK pathways. VKR activation was shown to also activate the Jnk MAPK pathway, potentially involved in oocyte migration. **(B)** Morphology of the reproductive organs of *S. mansoni* females is affected by *dsSmvkr* RNA interference. Worms were treated with control irrelevant **(A)** or *dsSmvkr* **(B)** dsRNAs (as described in Vanderstraete et al., 2014). Whole-mount preparations were stained with carmine red and examined by confocal laser scanning microscopy. In control worms (a), we observe immature oocytes (io) within the smaller, anterior part of the ovary and big and mature oocytes (mo) in the posterior part. The ootype (ot) contains a viable egg formed by one oocyte and vitelline cells. Treatment with *dsSmvkr* (b) induced a strong disorganization and size reduction of the ovary, as well as the abortion of egg formation. Scale bar: 20 μm.

and eggshell formation in these parasites (Knobloch et al., 2006; Buro et al., 2013). Moreover, diverse CTKs of *S. mansoni* (SmTK3, SmTK4, and SmTK6) were shown to form complexes susceptible to participate in RTK signaling in gonads (Beckmann et al., 2011).

The EGF receptor SER is transcribed in the vitellarium and ovary of schistosome females together with its potential substrate SmEps8 (EGF-R kinase substrate 8) and SmTK3, the CTK that was shown to play a dominant role in proliferation of vitelline cells (Kapp et al., 2004). Since SmEps8 interacts in yeast two-hybrid (Y2H) assays with SmTK3, this might indicate a possible role of SER and EGF pathways in reproductive activities (Beckmann et al., 2010a).

Insulin pathways, and especially the insulin-mediated PI3K pathway, play major roles in reproduction processes in many organisms (Graf et al., 1997; Brown et al., 2008). Moreover, the TOR (target of rapamycin)/S6K (p70 S6 kinase) pathway has

been identified as an essential nutrient-sensing tool regulating egg development under the control of insulin in mosquitoes (Arsic and Guerin, 2008). Limited information has been obtained concerning the importance of IR receptors in the development of schistosome gonads, except that vaccination of mice with the purified insulin-binding domain of SjIR2 provoked a retardation of adult *S. japonicum* growth and a substantial decrease of egg maturation and laying in parasitized animals (You et al., 2012). While transcriptome analyses did not highlight an overexpression of IR transcripts neither in *S. mansoni* (Nawaratna et al., 2011) nor in *S. japonicum* (Gobert et al., 2009) gonads, they indicated an overexpression of VKR-like transcripts in ovary and vitelline cells of *S. japonicum* (Gobert et al., 2009). In *S. mansoni*, quantitative-PCR results confirmed that both *Smvkr1* and *Smvkr2* genes were more actively transcribed in female worms than in males (Gouignard et al., 2012). By *in situ* hybridization, *Smvkr1* and *Smvkr2* transcripts were detected in testes but they were more abundant in ovaries, in which the expression profile of each *vkr* was noticeably different. *Smvkr1* transcripts were present mainly in the posterior part of the ovary that contains mature oocytes (in prophase I of meiosis) whereas *Smvkr2* transcripts were found in the anterior part of the organ containing immature oocytes. Q-PCR data obtained from isolated ovaries (Hahnel et al., 2013) indicated that both *Smvkr1* and *Smvkr2* transcripts were up-regulated strongly in the ovaries of sexually developed females as compared to the organs from virgin females issued from unisexual infections. Additionally, two RTKs of the FGFR family, SmFGFR-A and SmFGFR-B were found among the genes identified as up-regulated in female gonads after pairing (Hahnel et al., 2014, this issue). Analyses of isolated testes confirmed the presence of *Smvkr1* and *Smvkr2* transcripts in male reproductive organs and showed similarly their up-regulation in testes from males issued from bisexual infections (Vanderstraete et al., 2014). This indicated the particular importance of SmVKR receptors during the development and maturation of schistosome reproductive organs.

Recently, molecular partners of SmVKRs have been identified from Y2H screening of an adult *S. mansoni* cDNA library with active intracellular domains of VKRs as baits. The analyses of the resulting partners indicated similarities between VKR and IR pathways, which is in agreement with the identities already observed within the intracellular domains of these RTKs. All the assumptions made from the nature of these partners about the specificity of phospho-pathways elicited by VKR, were supported by further studies of VKR activation and signaling in *Xenopus* oocytes. It was shown that ligand-activated VKRs induced, similarly to the endogenous insulin-activated Xenopus IR, the phosphorylation of Erk1/2, Akt, and p70S6K (Vanderstraete et al., 2014). This indicated that VKRs were able to stimulate protein synthesis and cellular growth, as IRs do. The JNK pathway was activated by SmVKR1 but not by SmVKR2, corroborating Y2H screening results which showed a specific interaction of SmVKR1 with Rho1, Mek7, and PP2C (Vanderstraete et al., 2014). Since the JNK pathway has been shown to play a major role in oogenesis and meiosis resumption in *Caenorhabditis elegans* (Smith et al., 2002), in *Drosophila melanogaster*

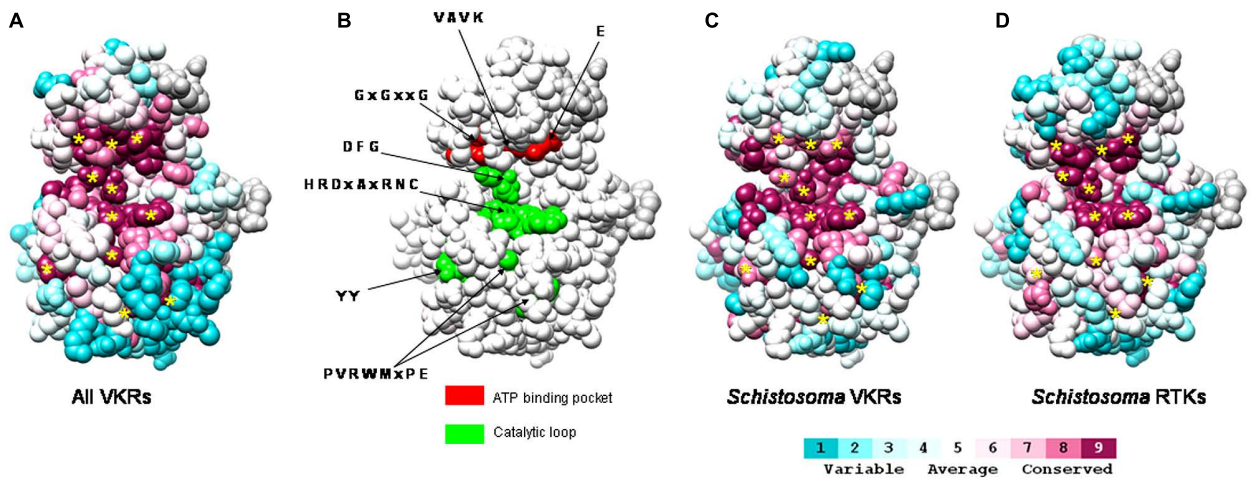

FIGURE 2 | Conservation of schistosome RTK tyrosine kinase catalytic sites. An evolutionary trace analysis of the conservation of residues in TK domains was performed using sequence alignment of **(A)** 40 VKRs already known in various invertebrate species (Vanderstraete et al., 2013a), **(C)** four VKRs of *Schistosoma* (SmVKR1 AAL67949.1 and SmVKR2 ADD91576.1, and two VKRs of *Schistosoma haematobium* Sha_103537 and Sha_104501), **(D)** various *Schistosoma* RTKs (EGF, insulin and VKR receptors) including SmVKR1, SmVKR2, the two ShVKRs, SmIR1 (AAN39120), SmIR2 (AAV65745.2), SjIR1 (ACT20714.1), SjIR2 (ACT20715.1), and SER (AAA29866.1). Visualization of the conservation was performed on the human IR TK crystal structure (PDB accession number 1IRK; Ahier et al., 2009). The alignment generated with ClustalW was submitted to the ConSurf website server (http://consurf.tau.ac.il; Landau et al., 2005). Conservation scores of each residue were calculated by taking into account the phylogenetic relationships among the sequences and the similarity between the amino acids in the alignment. Conservation scores are according to a color scale from variable (blue) to conserved (purple) residues. In **(B)**, are indicated the crucial residues of the ATP binding pocket (red) and of the catalytic loop (green) required for kinase activity. The evolutionary trace analyses revealed that these crucial residues (indicated by * in **A,C,D**) are highly conserved among all VKRs **(A,C)** and among the panel of *Schistosoma* RTKs (EGF, insulin, and VKR receptors) from the three human species (*mansoni, haematobium,* and *japonicum*).

(Sackton et al., 2007), and in mammals (Huang et al., 2011; Chuderland et al., 2012), it was postulated that it could be used as a pathway by SmVKR1 to influence oocyte maturation. Furthermore the implication of schistosome VKRs in oogenesis and spermatogenesis was demonstrated by RNA interference. SmVKR silencing led to an important disorganization of the antero-posterior structure of the ovary and the knock down of *Smvkr1* resulted in the accumulation of big oocytes in the ovary and the absence of egg formation (**Figure 1B**). In male testes, silencing of both *Smvkr* provoked a decrease of cell density within testicular lobes and paucity of sperm, confirming the potential importance of VKRs in reproduction processes (Vanderstraete et al., 2014).

RTKs AS POTENTIAL TARGETS FOR THE CONTROL OF SCHISTOSOMIASIS

Given the oncogenic role of aberrant signaling from RTKs in humans, these receptors have become attractive therapeutic targets. This led to the generation and the use in therapy of a large number of TK inhibitory compounds. Some of them were named as "tyrphostins" (TYRosine PHOSphorylation INnhibitors; Levitzki and Mishani, 2006), compounds that are able to inhibit multiple RTKs. We have seen that growth factor receptor and TK signaling molecules likely represent key molecules for the development and reproductive activity processes in schistosome worms. Consequently, these molecules are considered as potential targets for novel therapies against schistosomiasis today (Dissous et al., 2007; Dissous and Grevelding, 2011). IR inhibitors [tyrphostins AG1024, AG538, and HNMPA-(AM)3] can potentially affect survival of *S. mansoni* (Ahier et al., 2008) and *S. japonicum* (You et al., 2010) adult worms by blocking the uptake of glucose, an essential nutrient for schistosomes. Imatinib (Gleevec), used in the treatment of multiple cancers but targeting notably the chimeric oncogene BCR-Abl responsible for chronic myelogenous leukemia (Manley et al., 2002) has been described for its fatal impact on morphology, pairing stability, and survival of adult *S. mansoni in vitro* (Beckmann and Grevelding, 2010). Other TK inhibitors have also revealed a potential usefulness for the prevention of egg production by schistosomes. The Src kinase inhibitor, Herbimycin A, was demonstrated to block mitotic activity, expression of eggshell protein gene and egg production in *S. mansoni* female worms, preferentially inactivating the parasite Src-related SmTK3 and causing its degradation (Knobloch et al., 2006). The Syk inhibitor Piceatannol blocked the kinase activity of SmTK4 of *S. mansoni* and reduced egg production by female worms *in vitro* (Beckmann et al., 2010b). Furthermore, the angiokinase inhibitor BIBF1120 was shown to block the activities of the FGFR receptors SmFGFR-A and SmFGFR-B from *S. mansoni* leading to severe effects on the morphology of gonad tissues and the gastrodermis, on reproduction leading to reduced egg production, and finally on worm vitality (Hahnel et al., 2014, this issue).

More recently, tyrphostin AG1024 emerged as a potent drug molecule that caused dramatic effects both on the viability of larvae of *S. mansoni* and on the fertility of adult worms. The remarkable efficacy of this TK inhibitor (used at μM doses) on parasites was shown to be due to its dual action on the parasite IR and VKR kinases that contain similar catalytic domains (Vanderstraete et al., 2013b). **Figure 2** illustrates the conservation of the catalytic pocket of RTKs characterized in the three human schistosome species. Using a sequence alignment of the TK domain of the various parasite RTKs and the crystal structure of the TK domain of the human insulin receptor, an evolutionary trace analysis revealed that in kinase domains the residues were highly conserved, and particularly those composing the catalytic loop and the ATP-binding site essential for kinase activity (**Figures 2B–D**). These residues were also identical among all the VKRs characterized in other invertebrate species (**Figure 2A**). The conservation of the motifs essential for TK activity in the various schistosome RTKs is very likely accounting for the efficacy of AG1024 to inhibit simultaneously schistosome IR, VKR but also EGFR kinase activities. This was confirmed by the high sensitivity of all these recombinant parasite kinases to AG1024 shown in kinase assays (Dissous et al., 2014b; Vanderstraete et al., 2014). In females of *S. mansoni* treated with AG1024, important size reduction and disorganization of the ovary were observed together with an inhibition of egg production. AG1024 also affected spermatogenesis in males, and all these data confirmed the potential of AG1024 to affect schistosome reproduction by targeting multiple RTKs. The implication of these RTKs in gametogenesis and reproduction processes are valuable reasons to consider them as interesting targets in new control programs, with the main advantage in the case of VKR that their counterparts are absent from the vertebrate host kinase panel. Structural divergences between catalytic domains of host and schistosome RTKs should now be exploited for the design of molecules able to target schistosome RTKs without affecting human host kinases. This should require for the parasite RTK kinase domains the obtention of X-ray crystal structures that would serve as the basis for designing new pharmaceuticals against schistosomiasis. Alternatively, the use of small molecules as antagonist ligands of the VFT domains of schistosome VKRs should be efficient to interfere specifically with VKR pathways and parasite reproduction.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 May 2014; accepted: 03 July 2014; published online: 18 July 2014.
*Citation: Morel M, Vanderstraete M, Hahnel S, Grevelding CG and Dissous C (2014) Receptor tyrosine kinases and schistosome reproduction: new targets for chemotherapy. Front. Genet. **5**:238. doi: 10.3389/fgene.2014.00238*
This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Genetics.

frontiers in GENETICS

ORIGINAL RESEARCH ARTICLE
published: 16 July 2014
doi: 10.3389/fgene.2014.00207

Exposure to hycanthone alters chromatin structure around specific gene functions and specific repeats in *Schistosoma mansoni*

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Schistosoma mansoni is a parasitic plathyhelminth responsible for intestinal schistosomiasis (or bilharzia), a disease affecting 67 million people worldwide and causing an important economic burden. The schistosomicides hycanthone, and its later proxy oxamniquine, were widely used for treatments in endemic areas during the twentieth century. Recently, the mechanism of action, as well as the genetic origin of a stably and Mendelian inherited resistance for both drugs was elucidated in two strains. However, several observations suggested early on that alternative mechanisms might exist, by which resistance could be induced for these two drugs in sensitive lines of schistosomes. This induced resistance appeared rapidly, within the first generation, but was metastable (not stably inherited). Epigenetic inheritance could explain such a phenomenon and we therefore re-analyzed the historical data with our current knowledge of epigenetics. In addition, we performed new experiments such as ChIP-seq on hycanthone treated worms. We found distinct chromatin structure changes between sensitive worms and induced resistant worms from the same strain. No specific pathway was discovered, but genes in which chromatin structure modifications were observed are mostly associated with transport and catabolism, which makes sense in the context of the elimination of the drug. Specific differences were observed in the repetitive compartment of the genome. We finally describe what types of experiments are needed to understand the complexity of heritability that can be based on genetic and/or epigenetic mechanisms for drug resistance in schistosomes.

Keywords: *Schistosoma mansoni*, resistance, induction, hycanthone, oxamniquine, epigenetics, repetitive sequences

INTRODUCTION

Schistosoma mansoni, a platyhelminth trematoda parasite, is the causing agent of intestinal schistosomiasis (or bilharzia). The parasite has a heteroxenous life cycle with two hosts and three main developmental stages. The cycle begins when eggs come in contact with fresh water and hatch, releasing a free-swimming larva known as miracidium. This larva infects the intermediate host (a freshwater snail, usually of the *Biomphalaria* genus). Inside the mollusk, it will transform into primary and then secondary sporocysts and undergo asexual multiplication. A second type of free-swimming larvae, cercariae, is released and infects the definitive mammalian host (human or rodent) in which they will reach their adult form (worm) and perform sexual reproduction. Sexually mature schistosomes couples are located in mesenteric veins of the mammalian host. Females lay eggs with a characteristic lateral spine, which allow eggs to go through the wall of the intestine and be excreted in the feces. However, a large proportion of eggs is caught in the blood circulation and end up being trapped in the liver, leading to hepatic fibrosis. Bilharzia is an endemic disease in many areas in Africa and South America, infecting 67 million people and causing an important socio-economical burden (King, 2010). Since the beginning of the twentieth century, several schistosomicides were developed (Galdino da Rocha Pitta et al., 2013). Many of them were abandoned due to low treatment efficiency, as well as toxic and sometimes mutagenic side effects to the human host (Galdino da Rocha Pitta et al., 2013). Another major concern regarding schistomicides is the rapid appearance of resistances, both in laboratory strains and natural populations of the parasite (Bruce et al., 1987; Coles et al., 1987; Drescher et al., 1993; WHO, 1998). Please note that to avoid any language confusion, we use here the definitions of drug resistance and tolerance in schistosomes as given by Coles (2002), with the former being "*when any isolate has a significantly lower cure rate than the most sensitive isolate*" and the

later when the whole species is non-responsive to the drug. These factors left a single drug, praziquantel, as the current therapeutic alternative. As there is very little advance in the development of novel drugs against schistosomiasis, it becomes crucial to deepen our understanding on which molecular and evolutionary mechanisms can lead to schistosomicides resistance. Such knowledge could allow to either avoid the rapid appearance of resistances previously seen in *S. mansoni*, or to find ways to circumvent it.

Various drug resistances in schistosomes were studied through the past decades, but one of the best documented, but also most intriguing one, is against hycanthone. This molecule is not used anymore in treatments, and has been commercially discontinued, but was subjected to many research works from different laboratories. First evidences of resistant worms to this molecule were published in the early 1970's (Rogers and Bueding, 1971). It was later demonstrated several times that schistosomes were in general cross-resistant or cross-susceptible to both hycanthone and another schistosomicide, oxamniquine (Jansma et al., 1977; Cioli and Pica-Mattoccia, 1984; Dias and Olivier, 1985; Bruce et al., 1987). Oxamniquine was often used as a proxy for hycanthone, as the later is not commercially produced anymore and was toxic to the host to a certain degree. Two laboratories obtained stable resistant lines of schistosomes by injecting infected mice with a single curative dose (i.e., killing >90% of the worms in susceptible lines, 80 mg/kg) repeated over 3–5 successive generations of the parasite (Cioli and Pica-Mattoccia, 1984; Dias and Olivier, 1985). In these lines, resistance was heritable and stable even in the absence of drug pressure over 9–30 generations (Dias and Olivier, 1985; Cioli et al., 1989; Drescher et al., 1993). Classic genetic crosses between sensitive and resistant strains, as well as genetic complementation demonstrated that a single recessive autosomal locus codes for the hycanthone/oxamniquine resistance in two strains, MAP (named from the initials of the patient from which it was isolated) and Baltimore Rome Resistant (BRR) (Cioli and Pica-Mattoccia, 1984; Cioli et al., 1989, 1992; Pica-Mattoccia et al., 1992b, 1993). Similar results were also obtained *in vitro* by Coles and Bruce (1987). They cultured schistosomulas (an immature developmental stage occurring once cercariae have entered the mammalian host) under 240 p.p.m. of oxamniquine, a lethal concentration for 99.8% of the population. Survivors were injected in mice, and their progeny was not responsive to very high concentrations (500 mg/kg) of the drug. Experiments with tritiated hycanthone and oxamniquine demonstrated that the molecules were absorbed by all worms, but did not persist in resistant worms, while they were found to be covalently bound to DNA, RNA, and proteins in the sensitive strains (Pica-Mattoccia et al., 1988, 1989). Biochemical characterization showed that an enzymatic activation, probably by a sulfotransferase (Pica-Mattoccia et al., 2006), was needed by both oxamniquine and hycanthone to be lethal to the worm. Prior to its formal characterization, Pica-Mattoccia et al. (1992a) were able to biochemically isolate a fraction containing this enzyme from ground sensitive worms. They showed that resistant worms were killed when exposed *in vitro* to either hycanthone or oxamniquine and to the sulfotransferase-containing fraction from sensitive worms, while unaffected when exposed to one of the drugs alone. In the same article, the authors established that adult male worms, that are more sensitive to the drugs than females, have a higher enzymatic activity than females and immature stages (mostly unaffected by the drugs at the classical therapeutic concentration, and having a very low enzymatic activity). A difference in the enzyme specificity might also explain why hycanthone, but not oxamniquine, is efficient against *Schistosoma haematobium* (Pica-Mattoccia et al., 1997). The sequencing of the genome of *S. mansoni* (Berriman et al., 2009) provided new opportunities of investigation, and Valentim et al. (2013) recently used linkage mapping methods to identify the gene responsible for resistance in the HR and MAP strains. Candidate genes were screened using recombinant proteins, RNAi, and protein crystallography. The authors showed that it was indeed a sulfotransferase that was responsible for the resistance. They identified its coding gene (Smp_089320) and the non-synonymous nucleotide substitutions causing the loss of function of the enzyme in resistant lines MAP and HR, thus clearly establishing the mechanism of resistance in the studied lines (see Figure S1 in Valentim et al., 2013 for the mode of activation of oxamniquine).

Nevertheless, there are several observations that suggested early that alternative mechanisms might exist. For instance, Jansma et al. (1977) described three different ways to induce resistance by collecting the progeny of worms after a single injection of 60 mg/kg of hycanthone to the rodent host 54–70 days post infection (Type I), or 27–29 days post infection when worms are still at an immature stage (Type II), and from hosts infected by a single sex and then re-infected 2–58 weeks later by cercariae of the opposite sex (Type III). In all three cases, parents were all susceptible to the drug while a high percentage of the progeny survived the administration of a curative dose of hycanthone. Resistance was shown to be metastable to various degrees (depending on the type of induction used) through 10–21 generations. By metastable, we mean that from one generation to another, the percentage of worms surviving hycanthone treatment was highly fluctuant and did not follow a classical Mendelian inheritance pattern. For example, it was observed at several occasions by Jansma et al. (1977) that while a parental generation was completely resistant (100% survivors), a high proportion of the direct progeny (sometimes up to 75%) was found sensitive. Sometimes, the amount of resistant parasites would go up again a couple of generations later. These results are in complete contrast of the work mentioned above where classical recessive monogenic pattern for the inheritance of the resistance was observed. The work of Jansma was partly reproduced by Brindley et al. (1989). They were able to achieve Type II induction with lines of schistosomes deriving from Jansma's isolate.

As these observations do not fit the monogenic resistance model recently described, we reasoned that environmentally triggered drug resistance with metastable inheritance is a legitimate candidate for an epigenetically based phenotype. We use here a more narrow definition of epigenetics, meaning heritable changes in gene expression, without changes in the DNA sequence (Wu and Morris, 2001). They can be physically based on chemical modification of the DNA (e.g., methylation of cytosines), changes of proteins interacting with DNA (e.g., histone modifications), as well as nuclear localization of chromosomes, and short untranslated RNA playing a role in post-transcriptional silencing of

genes (Aguilera et al., 2010). There are numerous examples in the literature on how environmental factors, such as nutrition, pollution, temperature, and others, can have an influence on epigenetic control (Feil and Fraga, 2012). A drug treatment could, for example, induce an epimutation, silencing the gene coding for the enzyme responsible of the bio-activation. This epimutation would be more or less stable and/or inherited to progeny given the parasite strain (as the phenotype was previously described to be either stable or metastable through generations depending on investigators and strains). This type of transient transgenerational modification was initially also called "Dauermodifikation" (Jollos, 1921).

From this hypothesis, we decided to revisit the historical data and to complement it with our own study. DNA methylation has been found to regulate oviposition (Geyer et al., 2011). However, it is globally at a very low level through the genome (Raddatz et al., 2013), and therefore we decided to focus on another epigenetic information carrier, histone modifications. We decided to use a Brazilian (SmBre) and Guadeloupian (SmGH2) strain. Both strains were previously pool-sequenced (Clément et al., 2013) and we found that they were both of low genetic diversity (Tajima's pi of 2.10^{-4} for SmGH2 and $1.8.10^{-4}$ for SmBRE) and do not have any coding polymorphism in the gene (Smp_089320) uncovered by Valentim et al. (2013). Even if there is an almost uniform genetic background within the strains, we could not, at this stage completely rule out that an induction phenotype could come from the selection of a novel resistance allele in some schistosomes and not an epigenetic change. The debate whether heritable characters are induced by the environment or whether they occur randomly and are subsequently selected is not new. In 1951, in order to differentiate between "spontaneous (genetic) mutation and natural selection" and "directed mutation" or induction, the Lederbergs had designed their now famous experiment using replica plating of bacterial colonies from non-selective support to Petri dishes with selective media (Lederberg and Lederberg, 1952). Their work gave results in favor of the spontaneous mutation, i.e., a genetic variability (diversity) based mechanism. We reasoned that it would be in principle possible to reproduce the classical Lederberg experiment, but this time using clonal populations of *S. mansoni* instead of bacterial clonal colonies.

Our results show that one of the strains, SmBre, is resistant to hycanthone in the absence of mutations in Smp_089320. After exposure to the drug, the chromatin structure is altered around genes belonging to gene ontology (GO) groups involved in catalytic activity and response to stress. This finding suggests that epigenetic mechanisms could be responsible for adjustments of the parasites metabolism leading to temporary resistance, thus reconciliating the seeming contradictions between stable and metastable types of the phenotype.

MATERIALS AND METHODS

MAINTENANCE OF THE PARASITES

The *Schistosoma mansoni* strains SmBre and SmGH2 were originally sampled in Brazil and Guadeloupe, respectively. Both strains were maintained in their sympatric intermediate host strain BgBre and BgGua of the mollusk *Biomphalaria glabrata* and *Mus musculus* as definitive vertebrate host. SmBre was originally sampled in the 1960s in Recife, and SmGH2 was isolated on Nov. 10th, 1983 from a patient in Ste. Rose (Guadeloupe, French West Indies) (Pers. Commun. A. Théron).

PRELIMINARY DRUG SCREENING OF THE STRAINS

Both SmBre and SmGua were screened for resistant individuals by first infecting five mice (for each strain) with 70 mixed-sex cercariae. Mollusks from which cercariae were collected were initially infected with 20 miracidia each, and hence cercariae shed are of multiple genotypes. Mice were injected 62 days later with a dose of 60 mg/kg of hycanthone (similar to the one used by Jansma et al., 1977). Eight weeks later, the mice were sacrificed by a lethal intraperitoneal injection of sodium pentobarbital, and surviving adult worms were recovered by retrograde perfusions of the hepatic portal system with citrate (7.5%) saline (8.5%) solution administrated through the left ventricle (Duvall and DeWitt, 1967). Worms trapped in the liver or mesenteric system were collected after excising these organs.

LEDERBERG-LIKE EXPERIMENT

Biomphalaria glabrata BgBre individuals were infected with single miracidium from SmBre. The experiment was repeated with several different *B. glabrata* BgBre host individuals, each one infected with a different *S. mansoni* clone (each miracidium came from different parents). We genotyped with sex markers cercariae shed by each *B. glabrata* following Beltran et al. (2008), and we selected five mollusks infected with male parasites. We chose to work only on male schistosomes as it has been shown that they are more sensitive to hycanthone (Pica-Mattoccia et al., 1992a) than females, and to avoid sex-based bias in the epigenome analysis. From each snail, we infected 10 mice with 70 cercariae each. Sixty-two days later, half of the mice were injected with a dose of 60 mg/kg of hycanthone, and the other half with Ringer's solution as a negative control. Fifty-seven days after injection, we perfused the mice following the method of Duvall and DeWitt (1967) to collect control and surviving, resistant worms. A graphical abstract of the methodology is on **Figure 1**.

CHROMATIN IMMUNOPRECIPITATION AND SEQUENCING

Native ChIP was performed following the protocol developed for *Schistosoma mansoni* by Cosseau and Grunau (2011) (also available online at http://methdb.univ-perp.fr/cgrunau/methods/native_chip_sm.html) on a pool of six resistant and a pool of 10 control adult worms. Immunoprecipitation was performed using the following antibodies: H3K4me3 (Millipore, cat# 04-745 lot# NG1680351, 4 µl per reaction), H3K9Ac (Millipore, cat# 07-352 lot# DAM16924924, 8 µl per reaction), H3K9me3 (Abcam, cat# ab8898 lot# 733951, 4 µl per reaction), and H3K27me3 (Diagenode, cat# pAb-069-050 lot# A29900242, 8 µl per reaction). ChIP products were sequenced as single-end 50 bp reads on an Illumina HiSeq 2000 at the Montpellier GenomiX facility (http://mgx.cnrs.fr). We performed PCR amplification of the two exons of Smp_089320 on control and resistant ChIP products to test if a possible somatic mutation had occurred in that gene. We used Promega GoTaq Flex (cat# M8291) at 3 mM $MgCl_2$ and with the following PCR conditions: initial denaturation 2 min at 95°C followed by 35 cycles of denaturation 95°C

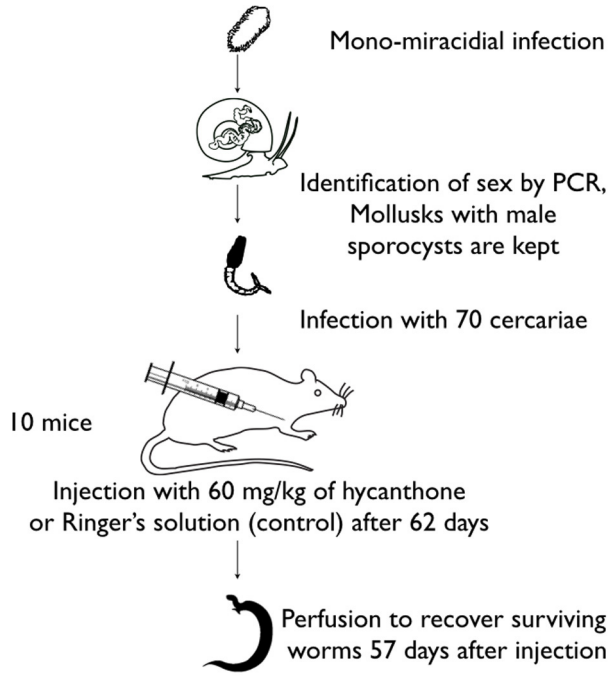

FIGURE 1 | Graphical abstract of the methodology used for the Lederberg-like experiment.

for 30 s, annealing 62°C for 30 s and elongation 72°C for 1 min. A final extension of 72°C for 5 min completed the reaction. We used two primer pairs to amplify exon 1 (Fwd1 tccacctctctctcactcaatg, Rev 1 ccacatgttggtaaatccgta, Fwd2 gctggtctaccgagaactgg and Rev2 tcccgtagaaaaccaactcg) and one pair for exon 2 (Fwd agtccattcattcaatgtttcaa and Rev caatccacaaatccccattc). PCR products were gel purified using Promega Wizard SV Gel and PCR Clean-Up System (cat# A9282) and sequenced at the Genoscreen facility (http://www.genoscreen.fr/).

QUALITY CONTROL AND ALIGNEMENT

All data treatment was carried out under a local galaxy instance (Goecks et al., 2010) (http://galaxy.univ-perp.fr). We used the FASTX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) for verification of the reads quality. Read quality was judged sufficiently good (the majority of reads showed a fastqsanger quality score $\geq$24 for all positions). For the analysis of unique sequences, reads were aligned on the *Schistosoma mansoni* genome assembly v5.0 (Protasio et al., 2012) with Bowtie2 (Langmead and Salzberg, 2012) using parameters—sensitive -k 2. Reads with unique match were filtered with samtools (Li et al., 2009) (samtools view -hS -q 255). For peak calling, an equal number of randomly selected reads was chosen between the treated and control conditions (H3K4me3 = 2.9 million, H3K9Ac = 23 million, H3K9me3 = 21 million, H3K27me3 = 17.9 million). Peak identification was performed using PeakRanger v1.16 (Feng et al., 2011) with *P*-value cut off 0.0001, FDR cut off 0.01, Read extension length 200, Smoothing bandwidth 99 and Delta 1. Wiggle files generated by PeakRanger where uploaded in our local instance of GBrowse (Stein et al., 2002) (http://genome.univ-perp.fr) for visualization. For the analysis of repetitive sequences, reads were aligned with Bowtie2 evoking parameters—sensitive -k 5. The 3,145 repetitive consensus sequences used as reference in this study were obtained from a previous analysis done with the same strain SmBre (Lepesant et al., 2012). From the resulting SAM file, we choose the position with the highest score for each read. Normalization of SAM files was done by randomly removing a given portion of the counts to achieve the same effective library size (H3K4me3 = 0.7 million, H3K9Ac = 9.8 million, H3K9me3 = 10.8 million, H3K27me3 = 13.9 million). Elements that showed differences in histone modification levels where annotated using the ABBlast search engine within the RepeatMasker program (Smit et al., 2010). The search was performed for both nucleotide and protein databases. Sequences without match where compared against the nucleotide database of NCBI using the blastn search engine and against the non-redundant protein database using blastx (Altschul et al., 1990).

COMPARATIVE ANALYSIS

To find differences in the histone mark pattern between control and treated conditions, we used the Pyicoenrich function (with –binsize 0.3) from Pyicos v2.0.6 (Althammer et al., 2011). Visual inspection was performed on all the regions detected by the software with a z-score above 7. The GO enrichment analysis was performed using BLAST2GO (Conesa et al., 2005). For the analysis of significant differences in histone isoform enrichment in repeats, normalized read counts were compared using the DESeq package of the R software (Anders and Huber, 2010). This package uses the negative binomial distribution to calculate the *P*-value for each element between two compared samples and find elements that present significant differences in their enrichment levels.

GO ENRICHMENT ANALYSIS

The 60 genes for which chromatin structure differences had been detected were annotated using Blast2GO (Conesa et al., 2005). In order to check if GO terms would be overrepresented in this group of genes, 60 transcripts were randomly sampled out of 41,669 transcripts generated by a combination of RNA-Seq data and Sanger annotation. RNA-Seq data were generated from several biological replicates and these data will become available when the analysis is fully finished. The random samples were annotated as above and GO enrichment analysis was performed using Fisher's exact test implemented in Blast2GO and a *p*-value of 0.2 as cutoff. Random sampling was repeated 3 times.

RESULTS

A STRAIN WITH SENSITIVE GENOTYPE DISPLAYS HYCANTHONE RESISTANCE

We compared the number of worms recovered in mice injected with hycanthone to those recovered from control mice. We found 25 (36%) surviving worms, perfused from 5 mice, in the Brazilian strain SmBre, despite the fact that it has the sensitive genotype for Smp_089320. The Guadeloupian strain SmGH2, however, is completely sensitive to hycanthone, i.e., no survivors were found in any of the five infected and treated mice.

CLONAL SCHISTOSOMES RESPOND DIFFERENTLY TO HYCANTHONE TREATMENT

Following the discovery of hycanthone resistant individuals within the SmBre population, we adapted the Lederbergs' experiment to our schistosome model. To do so, we infected five *Biomphalaria glabrata* with a single, genetically distinct, SmBre miracidia. Cercariae shed by each mollusk are genetically identical. We then infected 10 mice with 70 cercariae. Half of the mice were then treated with an injection of hycanthone similar to the one described by Jansma for Type I induction (Jansma et al., 1977), and the other half was injected with Ringer's solution, as negative controls. As we excluded the possibility of a somatic mutation in Smp_089320, similar to the classical replica plating experiment, two possible outcomes can be imagined: (1) if a random mutation had occurred in one of the clones that conferred resistance to hycanthone (pre-existence), then only cercariae from this very snail would produce resistant flukes because they are genetically identical (**Figure 2A**) (2) if the phenotype is induced through the treatment with a certain probability, we would observe resistance randomly associated with different mice hosts and without correlation with the particular snail host (similar to Lederbergs' bacterial colony, **Figure 2B**). We later perfused the mice to look for control and surviving, resistant worms. The results of our experiment are shown in **Figure 3**. In summary, while the vast majority of mice were exempt of parasites after hycanthone injection, we found surviving worms in six different rodents, belonging to three different *S. mansoni* genotypes (B–D). We did not find any worm originating from genotype A and E after the treatment. In contrast, under the control condition, we recovered an average of 30 $\pm$ 5 worms (confidence interval at α 5%) per mice for the five genotypes. Sequencing of Smp_089320 in resistant worms did not show any of the three mutations identified by Valentim et al. (2013) or any new polymorphism.

CHROMATIN STRUCTURE CHANGES ASSOCIATED WITH THE RESISTANT PHENOTYPE

We performed ChIP-Seq on the pool of the six hycanthone resistant worms, as well as on control worms. We targeted four modifications of histone H3: tri-methylation of lysine 4 [H3K4me3], tri-acetylation of lysine 9 [H3K9ac], tri-methylation of lysine 9 [H3K9me3], and tri-methylation of lysine 27 [H3K27me3]. The first two are associated with euchromatin (relaxed DNA structure allowing transcription) and the later two are associated with heterochromatin (condensed DNA structure impeding transcription). Around Smp_089320 none of the marks showed enrichment. We then searched for variations in the chromatin structure at the genome level, and a bioinformatic analysis, followed by visual inspection, was able to detect differences in the histone modification profiles between hycanthone resistant and control worms. We found 47 differences for H3K4me3, 43 of which being located at the transcription start site of genes. This corresponds roughly to 0.6% of the total peaks detected for this histone mark. For H3K9me3, 12 differences were spotted (0.012% of total peaks), mostly in introns and within 2 kb upstream and downstream of genes. We identified five differences for each of the other two marks (0.007% and 0.005% of total peaks for H3K9Ac and H3K27me3, respectively), the majority being outside of genes. A list of the regions and associated genes in which we found differences is in Supplementary Table 1.

CHROMATIN STRUCTURE MODIFICATIONS ARE PREFERENTIALLY ASSOCIATED WITH GENES RELATED TO CATALYTIC ACTIVITY

None of the genes in which differences were uncovered code for a sulfotransferase. Using a GO enrichment analysis, we did not find any clear pathway linking together the genes bearing chromatin structure changes. However, we found that the terms "transferase activity" (13 occurrences), "kinase activity" (8 occurrences) and "catalytic activity" (26 occurrences) and "response to stress" (5 occurrences) were overrepresented in the panel of genes with epimutations.

CHROMATIN STRUCTURE MODIFICATIONS IN REPETITIVE SEQUENCES

We identified, in 14 repetitive sequences, statistically significant changes in chromatin structure. This is very few (0.44% of repeats) showing that globally the chromatin remains stable. Interestingly, changes in H3K4me3 and H3K9ac always decrease

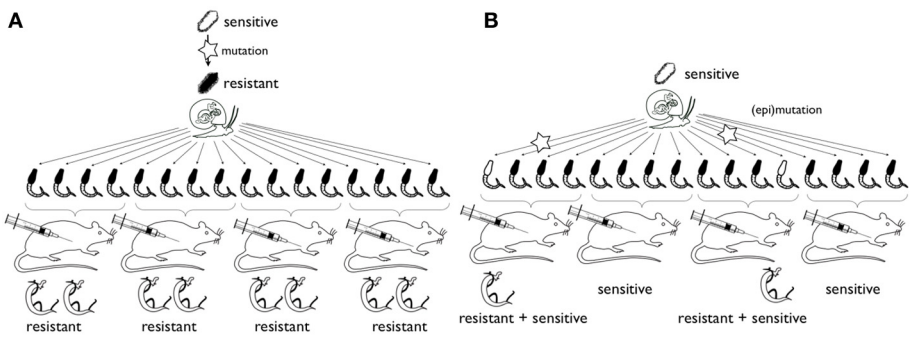

FIGURE 2 | Schematic representation of the experimental strategy for study of the origin of hycanthone resistance. Hypothesis **(A)** rare mutations (star) in miracidia (top) confer resistance to hycanthone. All offspring will be resistant (or sensitive, not shown). Hypothesis **(B)** epimutations or mutations occur during clonal amplification of cercaria in the snail host. Only few adult worms will be resistant. In the case of epimutation, in subsequent generations reversal of the phenotype would occur (and was described in the past) and a chromatin structure changes can be detected. In case of mutation, no reversal occurs and mutations should be detected in the locus of interest.

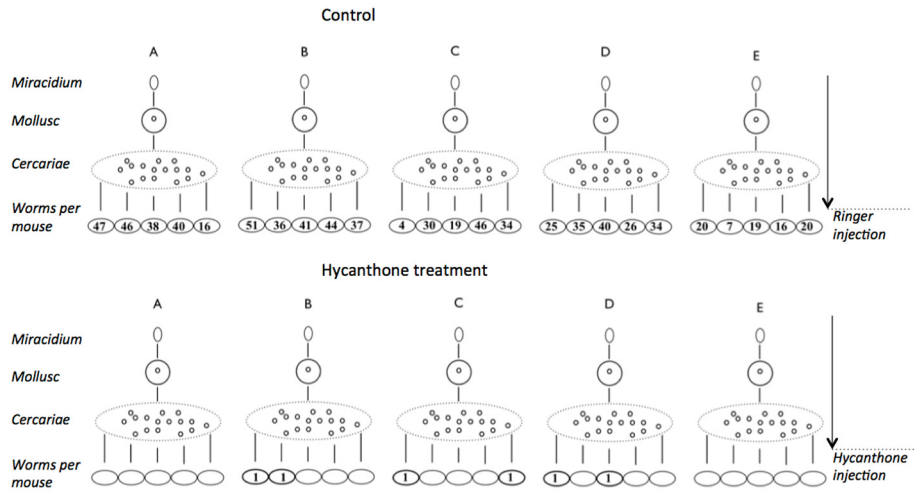

FIGURE 3 | Results of the experiment on induction of hycanthone resistance using mono-miracidial infections. Five mollusks were infected using a single miracidium (genotypes A–E). Cercariae shed from a single mollusk are clonal and were used to infect 10 mice. Half of the mice were injected with a control Ringer's solution and the other half with a therapeutic dose of hycanthone. Mice were later perfused and worms were counted. In the hycanthone condition, six worms of three genotypes (B–D) were recovered from six mice, while an average of 30 worms per mouse were found in the control condition.

in the treated worms while H3K27me3 increases, indicating a tendency toward heterochromatization. No changes occur in H3K9me3. H3K4me/H3K9ac modifications are observed in dispersed repeats that belong to the LINE/LTR classes. In contrast, H3K27me3 becomes enriched in simple repeats that are organized in blocks (Supplementary Table 2) sometimes covering the entire length of large contigs for which chromosomal location is unknown.

DISCUSSION

When screening the literature on hycanthone resistance, we were surprised to see such divergent results between the different research groups. On one hand, there is clearly established monogenic, stably inherited, resistance, and on the other, there is an inducible resistance, appearing from sensitive parents, and not inherited in a Mendelian fashion. We took a deeper look at the methodology employed by the all the research groups, and their interpretation of data, with the hope of finding variations in the procedures, or in the analysis methods, that could, at least partly, explain the discrepancies between the observations and conclusions.

We first tried to track back the origins of the strains that were employed in the various studies on hycanthone/oxamniquine resistance (**Table 1**). Our conclusion is that very few strains were used in these studies, and most of them had been maintained in laboratory for a very long time, probably reducing the genetic diversity of the strains. We identified six resistant strains in the literature, and four of them derived from the M strain used in Jansma et al. (1977) for the first induction experiments. We also found four main sensitive strains. At least four other strains (either resistant or sensitive) were punctually used and will not be discussed here (Bruce et al., 1987; Drescher et al., 1993; Pica-Mattoccia et al., 1993).

In his Type I induction, Jansma (Jansma et al., 1977) injected a dose of 60 mg/kg of hycanthone 54–70 days post-cercarial infection. He noted a marked reduction in the number of worms, but rarely a complete elimination. Surviving worms were unable to produce viable eggs for 4–8 months. After that period, eggs from which miracidia were hatched were collected to produce a F1. This first generation was treated with a therapeutic dose of hycanthone (80 mg/kg) and 94% of the worms were found to be resistant. Twenty-one successive generations were produced from this F1, and on several occasions, additional groups of mice were infected from cercariae batches shed on different days, by the same infected mollusks, leading to parallel generations known as branches. Resistance tends to decrease with generations in the main branch, and is highly variable between branches (some are totally resistant for several generations, some become completely sensitive, and others are fluctuant). Although it is not explicitly stated in the paper, it seems that progeny for generations F2–F21 was collected before the therapeutic drug injection used to estimate the percentage of resistant parent worms. Hence, there was no drug selective pressure from one generation to another, and that could partly explain the metastability observed by Jansma but not by other investigators, as suggested by Cioli and Pica-Mattoccia (1984). It is conceivable that Type I resistance was achieved not by induction, but through the selection worms with a resistant genotype, and as the induction dose is slightly below the therapeutic one (normally killing >90% of the parasites), there is possibility that some worms with a normally "sensitive" phenotype, escaped the drug. Cioli et al. (1992) observed that their resistant strain derived from Jansma's had a lower global infectivity (to mollusk and mammalian host) and a lower fertility (lower amount of eggs per females) than their sensitive strain (although this could be strain related, as the sensitive strain from Cioli is not from the same origin as Jansma's). In the hypothesis

Table 1 | Origin and resistance phenotypes of the most commonly used strains in the literature.

Strain	Origin	Resistance	Derived from?	Comment	References
M	Puerto Rico	Oxa/Hyc	–	Used for the three types of induction experiments	Jansma et al., 1977
BRR	Puerto Rico	Oxa/Hyc	M	Underwent drug selective pressure over 3 generations	Cioli et al., 1992; Pica-Mattoccia et al., 1992a,b, 1993, 2006
JHU	Puerto Rico	Oxa/Hyc	M	Successful Type II induction	Brindley and Sher, 1987; Brindley et al., 1989, 1991
H-30	Puerto Rico	Oxa/Hyc	M		Souza et al., 1988
HR	Puerto Rico	Oxa/Hyc	M		Valentim et al., 2013
MAP	Brazil, Minas Gerai	Oxa Partial to Hyc	–		Dias and Olivier, 1985; Bruce et al., 1987; Souza et al., 1988; Pica-Mattoccia et al., 1992a,b, 1993; Drescher et al., 1993; Valentim et al., 2013
SEN	Puerto Rico	None	–		Cioli, 1976
BH	Brazil, Minas Gerai	None	–	Isolated from an untreated patient in 1967	Dias and Olivier, 1986; Bruce et al., 1987
NMRI	Puerto Rico	None	–	Isolated in the 1940'	Fletcher et al., 1981; Brindley and Sher, 1987; Brindley et al., 1989
LE	Brazil	None	–		Valentim et al., 2013

Hyc, Hycanthone resistant; Oxa, Oxamniquine resistant.

that some "sensitive" worms survived the induction step and were mixed with selected resistant worms, this could explain why the resistance is does not follow a Mendelian inheritance. This cannot be the case in Type II, as immature worms have little or no sensitivity to hycanthone (induction was also successful with subcurative doses ranging from 3 to 60 mg/kg) and in Type III where the parents were not exposed to the drug before collecting the progeny. These observations lead us to think that the resistant phenotype in Type II and III could indeed be induced and transmitted to the progeny to a certain extent. Cioli and Pica-Mattoccia (1984), Cioli et al. (1989) and Dias and Olivier (1986) stated that they were unable to reproduce Jansma's results in inducing Type II or III resistance. Three other antihelminthics, oxamniquine, praziquantel, and oltipraz were also unsuccessfully tested with the hope of inducing Type II resistance and selecting a new line of resistant schistosomes (Dias and Olivier, 1986). However, we have a slightly different interpretation of the results from Dias and Oliver. In their induction protocol, mice were treated with subcurative dose of one of the three drugs, 26–30 days after cercarial infection (when schistosomula have a low sensitivity to both hycanthone and oxamniquine), and then divided into two groups. The first group was treated with a curative dose of drug, while the second was left as a control, and the eggs from that control group were used to restart a cycle of the parasite. Mice infected by the F1 cercariae from the control group were also divided into a trial group treated with a curative dose of the drug and a control group (which would serve to generate a F2). No resistant worms were found in the treated F1 and F2, but 8–12% where found in the parental generation (in which the induction step was performed). Admittedly, there was no inheritance of a resistance phenotype to F1 and F2, but the fact that survivors were found only in the parental generation which underwent the induction step let us believe that it was not as a failure at inducing resistance as described by the authors. In fact, only when induction was performed, some resistant worms were found. Type II was also successfully induced by the Brindley group (Brindley et al., 1989) in several lines of the parasite deriving from Jansma's isolate. However, while four strains tested in this article were described as sensitive by the authors, it is worth to mention that in some of the trials, up to 26% of the worms survived the hycanthone treatment (with or without preliminary Type II induction). Brindley also observed spontaneous occurrence of resistance in other strains with no induction, although he admitted that "*whether these fluctuations reflect true changes in the parasite genotype rather than experimental variation was not systematically investigated in this study*" (Brindley et al., 1989). This lead us to realize that for the majority of research groups, resistance was considered as achieved when at least 90% of the worm population survived a therapeutic dose. In several studies (Dias and Olivier, 1986; Brindley and Sher, 1987; Brindley et al., 1989; Drescher et al., 1993), various lines of schistosomes did not reach this resistance threshold (with or without induction and/or drug pressure over a couple of generations). They were considered as sensitive although in some cases there was a non-negligible percentage (3–26%) of worms surviving a therapeutic dose of either hycanthone or oxamniquine, strengthening our hypothesis that a mechanism following a non-Mendelian inheritance pattern is involved. In this context, we believe it is more appropriate to talk about the penetrance of the phenotype rather than talking strictly about sensitivity or resistance.

Also, Cioli and Pica-Mattoccia specifically mentioned that their approach was different from the Jansma/Bueding/Brindley group. Three generations of selection were necessary to obtain a phenotype that was stably inherited by the progeny (Cioli and

Pica-Mattoccia, 1984). The same observation was made by Dias and Olivier (1985), as in their experiments, resistance was stable only after the 5th generation (experiments were continued until 14th generation). Unfortunately, the data for the first 3–5 generations are not available but one can assume that incomplete penetrance of the phenotype was observed. These large differences in penetrance of resistance phenotypes (i.e., large dose dependency) in different isolates were also found by others (Coles et al., 1986; Kinoti, 1987) and also described in Pica-Mattoccia et al. (1993). Kinoti noted important variations in the dose of oxamniquine needed to eliminate 99% of the worms (ED99), in both laboratory and clinical trials. For example, East African isolates needed a dose of oxamniquine 200–250% higher than Puerto-Rican isolates to reach ED99 (Kinoti, 1987). In all publications, at least 3 generations of selection were necessary to produce stable resistance, but even then, phenotypic differences between the different resistance strains and most notably an exception to the Mendelian segregation pattern were observed. In the MAP strain for instance there was (1) a difference between heritability of hycanthone and oxamniquine resistance and (2) segregation did not follow the recessive pattern. Segregation could not be followed up because the females produced no eggs, i.e., no genetic analysis was possible ("*Thus, we are left with no obvious explanation for this unexpected partial exception to the overall recessivity of the MAP resistance*.") (Pica-Mattoccia et al., 1993). The reason for the incomplete penetrance in the first 3–5 generations cannot be determined with confidence from the published data alone. It might be that the initial strains showed a high degree of genetic diversity with only a few individuals carrying the mutation in the sulfotransferase gene. Molecular biology methods in *S. mansoni* research were not sufficiently advanced in that time to measure genetic diversity. From the few available data, however, it seems that the founder populations of the resistant strains were very small. Years of maintenance cycle in laboratories, as well as the possibility of a founder effect every time that the strains were transferred or shared with other laboratories let us think that the genetic diversity was probably low in the different strains. We also noted that most of the strains were from the same geographical areas (either Puerto Rico or the region of Minas Gerais in Brazil) and represent only a small share of geographic diversity of *S. mansoni.* Another reason for variability in penetrance could therefore be high phenotypic plasticity and/or variability in plasticity. Jansma's results were in favor of this latter mechanism, and he additionally described transgenerational plastic effects. It has also to be noted that while the work of Brindley and Jansma was performed using hycanthone, other research groups used oxamniquine. Although schistosomes where always cross resistant or cross sensitive to both molecules, hycanthone is known to have a mutagenic effect, and it could have played a role in the phenomenon of induction, which was never observed with oxamniquine.

In our own experiments, we worked with a strain bearing the sensitive genotype for the sulfotransferase gene previously identified as the source of hycanthone/oxamniquine resistance, but we still found in drug screening that in one strain (SmBre) about a third of the adult worms survived the treatment while another strain did not show survivors. Resistance of SmBre could be due to a mutation in a different locus and/or due to epimutations but this would still not explain Jansma's induction phenomenon. Therefore, we used a Lederbergs experiment to discriminate if the resistance was pre-existing or induced by the experimental conditions. Similar to the classical replica plating experiment, two possible outcomes could be imagined: (1) if the phenotype is induced through the treatment with a certain probability, we would observe resistance randomly associated with different mice hosts and without correlation with the particular snail host (similar to Lederbergs' bacterial colony) (2) if a random mutation had occurred in one of the clones that conferred resistance to hycanthone (pre-existence), then only cercariae from this very snail would produce resistant flukes because they are genetically identical. A caveat with the Lederberg experiment in organisms with epigenetic inheritance systems is the potentially heritable transcriptional and/or posttranscriptional level of diversity. It is possible that epimutations occur during clonal amplification of the cercariae (**Figure 2B**). Also, as long as somatic mutations cannot be excluded, mutation would do the same job. Both would mimic the outcome of the "induction" alternative. We found that not all the worms issued from the same miracidia (i.e., being genetic clones of each other), responded to the treatment the same way. While the vast majority was sensitive, we still found some resistant individuals. when interpreted in the context of Lederberg's experiment, would favor the directed (epi)mutation hypothesis. If we had selection of a pre-existing mutation or resistance phenotype, then we would have expected that all, or no, worms from a same genotype would present the same phenotype (resistance or sensitivity). From our results, we propose the hypothesis that epigenetics could be part of the mechanism underlying the resistance induction.

Our ChIP-Seq experiment provided evidences of epigenetic variation between resistant and control worms. Our analysis highlighted at least 64 chromatin structure changes between the two conditions, with an enrichment profiles in genes implicated in catabolic, detoxification, and transfer activity (Two examples can be seen in Supplementary Figure 1). No specific pathway linking these genes together arose, but it makes sense in the context of a response and the subsequent elimination to a drug to see these kinds of biological processes being involved (as stated before, hycanthone is known to be eliminated from resistant worms a certain amount of time after its penetration, while it is stuck in sensitive worms metabolism). It is therefore conceivable that the drug resistance observed in the surviving worms is due to epistatic interaction of multiple gene products rather than an (epi)mutation in a single locus. Another interesting finding is the presence of a differential H3K27me3 profile in the gene body of SmMRP1 (Multidrug Resistance Associated Protein 1, Smp_171740). In various species, including trematoda, this gene is part of a family of transporters playing a role in exclusion and elimination of xenobiotic compounds and metabolic toxins (Kasinathan et al., 2010). In schistosomes, it was demonstrated that SmMRP1 has higher levels of expressions in response to the widely used schistosomicide praziquantel, and possibly other drugs (Kasinathan et al., 2010; Greenberg, 2013). We found a significant enrichment in H3K27me3 around exon 21 of this

SmMRP1 in the control condition. Enrichment of this mark are usually associated with increases of facultative heterochromatin and gene repression (Trojer and Reinberg, 2007; Bannister and Kouzarides, 2011). Our current knowledge of the functional role of histone modifications in gene expression in *S. mansoni* does not allow us to affirm that the lower presence of this mark in the resistant worms would lead to an increased expression of this gene, but it stays a promising candidate for further experiments.

Fourty-seven percent of the genome of *S. mansoni* is composed of repeats (Lepesant et al., 2012). The function of these repeats is completely unexplored and, in general, they are not included in the analysis of molecular basis of phenotypic variation. We have shown earlier (Lepesant et al., 2012), that repeats can be of importance in schistosomes, and we decided to study their chromatin structure changes upon hycanthone treatment. Only a very small fraction of the repeats (0.4%) change their histone modification status. In contrast to other organisms, stress (in this case hycanthone treatment) does not seem to lead to an euchromatization and potentially mobilization of repeats but has the inverse effect (decrease of H3K9ac and increase in H3K27me3). Interestingly, the repeats in which H3K27me3 status changes are located in satellite-type blocks on specific locations on the genome. It is conceivable that they form knobs that separate genome domains and influence indirectly gene expression. Further work will be needed to understand the impact of these repeated regions on the definition of the chromatin structure in which they are included.

While this ChIP-Seq experiment brings new insight on epigenetic mechanisms and regulation in *S. mansoni*, it does have some limitations. In order to be as faithful as possible to Jansma's work to reproduce his Type I induction, we exposed the worms to hycanthone through an injection in the rodent host. However, this generates a degree of variability from one mouse to another, as each individual has a different metabolism, immune system, clearance rate, and other physiological factor influencing the exposure of the worms to the drug. Also, although cercariae issued from a same initial miracidium are theoretically clonal, genomic instability such as somatic mutation and recombination can occur during asexual life stages like sporocysts, as shown by Vieira et al. (1991), and Bayne and Grevelding (2003), and their impact on the phenotype should be taken into account. Nonetheless, we managed to recover worms that survived the hycanthone treatment at two occasions (initial drug screening and Lederbergs-like experiment), from a low genetic diversity strain and without any evidences that the gene uncovered by Valentim et al. (2013) was involved. This, in addition with the differences of chromatin structure observed between surviving and control worms lead us to think that induction of resistance in *S. mansoni* is not a myth after all. Nevertheless, we do not think that induction should be seen as a specific response of the epigenome to a certain environmental condition leading to over/under-expression of a specific gene. Instead, this induction phenomenon seems to come from the capacity of some strains, or some individuals within a strain, to extend their degree of plasticity beyond the genetically defined "default" state through chromatin structure changes. This allows them to overcome an environmental stress in the absence of a pre-existing stress-response phenotype. Depending on the length of the stress, we can imagine that the progeny can more or less efficiently inherit the epigenetic condition responsible for the phenotype until it is fixed in the population (through a DNA mutation for example). This fits well with theoretical framework developed recently in which heritable phenotypic variation is based on at least two components: the low-fidelity (epigenetic) system and the high-fidelity (genetic) system (Klironomos et al., 2013). In this model, upon changes in the environment, the low-fidelity system can generate new phenotypes that can explore the adaptive landscape. The phenotype is decoupled from the genotype and appears before the adaptive genotype. The low fidelity of the epigenetic system means the adaptative phenotype could theoretically be loss as quickly as it appears, unless a strong selective pressure is applied. This is consistent with the observations of the metastability of hycanthone resistance in absence of drug pressure (Jansma et al., 1977; Brindley et al., 1989), and the stability of the phenotype after 3–5 successive generations of schistosomes where under drug pressure (Cioli and Pica-Mattoccia, 1984; Dias and Olivier, 1985). Basically, epigenetic variation allows to "buy time" for a few generation (in this case, 3–5) until a stable, genetically transmitted phenotype occurs. To understand better the underlying mechanism, measuring the expression level of Smp_089320 and of candidate genes identified here would be informative in future studies.

Since hycanthone is not anymore used for treatment of bilharzia and the use of oxamniquine is depreciated (Galdino da Rocha Pitta et al., 2013), mechanism of resistance is more of academic interest. However, experience with hycanthone and oxamniquine shows that a new conceptual framework is necessary to design experiments that distinguish the part of genetics and epigenetics in resistance phenotypes. We believe that a standard procedure would be beneficial for the testing of resistance to current drugs, especially praziquantel, the only molecule actually used for mass treatment of schistosomiasis. Resistance to praziquantel shares striking similarities with early observations on hycanthone/oxamniquine. It can be induced through subcurative, but increasing doses of praziquantel over six-seven generations, and genotyping of two populations of schistosomes showed that there was no significant difference in the genotypes before and after drug treatment, meaning no selection of specific genotypes by the drug (Blanton et al., 2011; Greenberg, 2013). We think that a standard procedure for the next drugs should be similar to the one we used to detect resistant individuals from a clonal population. Mono-miracidial infection would be a good start, as it minimizes the genetic variation between individuals and allows to detect changes in the epigenotype. A higher number of mono-miracidial infections, using several strains of *S. mansoni* (as the induction seems to be strain dependent), and a larger number of clones (adult or schistosomula) from each mono-miracidial infection should be tested, as we still do not have a clear idea of the frequency of the phenomenon. Induction step can be done *in vivo* (for any of the tree types) but screening of resistant progeny has to be performed *in vitro*, as described by Coles and Bruce (1987) and Pica-Mattoccia et al. (1992b). This is mandatory to make sure that all parasites have the same exposure to the drug. To look for heritability, resistant worms can be surgically reinserted into new rodent hosts and allow to reproduce. It is critical to

have a standardized procedure to maintain the life cycle of the parasite (i.e., number of miracidia and cercariae, as well as their genotypes, used to infect the hosts) as it is difficult, if not impossible, in some older publications to find out if variation in the numbers of perfused worms come from effect of the drug or bias in infections. Using the same intermediate and final host is also important as they have an impact on the phenotype and life traits of the parasite. In the articles we reviewed, most experiments were done using the same final host (swiss albino mice), but sometimes hamsters were used, too. The geographical origin of the mollusk host is not always explicitly stated, and there are numerous evidences that it plays an important role in the parasite infection success and development (Theron et al., 1997). Although this was never explicitly discussed, it could be a cause to the contradictory results on hycanthone induction. Finally, both genomes and epigenomes of resistant worms would have to be compared to the ones of sensitive worms to figure out the origin of the phenomenon. Also, from our ChIP-Seq results, we saw that it is essential to keep a global, genome scale approach including repetitive sequences. The epimutations we found were distributed across the genome and are involved in various pathways. A candidate gene approach would probably have missed some of the variations we detected. Rather than only focusing on find a single gene responsible for the phenotype, we believe that the approach we describe would lead to a more complete answer on how schistosomes develop resistances.

ETHIC STATEMENT

The French Ministère de l'Agriculture et de la Pêche and French Ministère de l'Education Nationale de la Recherche et de la Technologie provided permit A 66040 to our laboratory for experiments on animals and certificate for animal experimentation (authorization 007083, decree 87–848 and 2012201-0008) for the experimenters. Housing, breeding and animal care followed the national ethical requirements.

AUTHOR CONTRIBUTIONS

David Roquis performed the literature review, achieved the bioinformatics analyses and wrote the manuscript. Julie M. J. Lepesant designed and performed the experiments, contributed to prepare the reagents and the materials. Emanuel Villafan and Cristina Vieira analyzed the data concerning repetitive DNA. Céline Cosseau conceived the experiments and took part in writing the manuscript. Jérôme Boissier contributed to drug treatments and acquisition of samples. Christoph Grunau conceived and designed the experiments, contributed to analysis tool preparation and bioinformatics analyses, and took part in writing the manuscript.

ACKNOWLEDGMENTS

We are grateful to Prof. Sam Loker (Albuquerque, NM, USA) who attracted our interest to induced hycanthone resistance and Prof. Michael J. Waring (Cambridge, UK) for sharing his precious stock of hycanthone. Without his help this work would not have been possible since the drug is not anymore commercially available. This work received support from the ANR 2010 BLAN 1720 01 (EPIGEVOL). The manuscript profited tremendously from the critical reading by two anonymous reviewers.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fgene.2014.00207/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 22 April 2014; accepted: 18 June 2014; published online: 16 July 2014.

*Citation: Roquis D, Lepesant JMJ, Villafan E, Boissier J, Vieira C, Cosseau C and Grunau C (2014) Exposure to hycanthone alters chromatin structure around specific gene functions and specific repeats in Schistosoma mansoni. Front. Genet. **5**:207. doi: 10.3389/fgene.2014.00207*

This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Genetics.

frontiers in GENETICS

ORIGINAL RESEARCH ARTICLE
published: 09 July 2014
doi: 10.3389/fgene.2014.00206

Evolutionary analysis of the cystatin family in three *Schistosoma* species

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The cystatin family comprises cysteine protease inhibitors distributed in 3 subfamilies (I25A–C). Family members lacking cystatin activity are currently unclassified. Little is known about the evolution of *Schistosoma* cystatins, their physiological roles, and expression patterns in the parasite life cycle. The present study aimed to identify cystatin homologs in the predicted proteome of three *Schistosoma* species and other Platyhelminthes. We analyzed the amino acid sequence diversity focused in the identification of protein signatures and to establish evolutionary relationships among *Schistosoma* and experimentally validated human cystatins. Gene expression patterns were obtained from different developmental stages in *Schistosoma mansoni* using microarray data. In *Schistosoma*, only I25A and I25B proteins were identified, reflecting little functional diversification. I25C and unclassified subfamily members were not identified in platyhelminth species here analyzed. The resulting phylogeny placed cystatins in different clades, reflecting their molecular diversity. Our findings suggest that *Schistosoma* cystatins are very divergent from their human homologs, especially regarding the I25B subfamily. *Schistosoma* cystatins also differ significantly from other platyhelminth homologs. Finally, transcriptome data publicly available indicated that I25A and I25B genes are constitutively expressed thus could be essential for schistosome life cycle progression. In summary, this study provides insights into the evolution, classification, and functional diversification of cystatins in *Schistosoma* and other Platyhelminthes, improving our understanding of parasite biology and opening new frontiers in the identification of novel therapeutic targets against helminthiases.

Keywords: schistosomiasis, proteinase inhibitor, phylogenomics, bayesian inference, function prediction

INTRODUCTION

Five species of the genus *Schistosoma* (Trematoda) are involved in the human infection, being the main etiologic agents of human schistosomiasis: *Schistosoma mansoni* and *Schistosoma japonicum* causing intestinal schistosomiasis, and *Schistosoma haematobium* causing urinary schistosomiasis. According to the World Health Organization, schistosomiasis is endemic in 77 countries, affects more than 200 million people worldwide, and other 779 million live in areas at risk of infection (WHO, 2012). Schistosomiasis control relies mainly on praziquantel treatment but its efficacy is limited. Furthermore, evidence of praziquantel resistant parasites was obtained in the laboratory and in endemic regions (Liang et al., 2003; Melman et al., 2009; Coeli et al., 2013). Hence schistosomiasis is still one of the most prevalent infectious and parasitic diseases worldwide being a major source of morbidity and mortality in developing countries.

The urgent need to develop novel drugs or a vaccine for *Schistosoma* species has encouraged an interest in the function prediction of relevant proteins for parasitism. The search for new drug targets based on evolutionary analyses using *S. mansoni* genomic/proteomic data has been performed (Silva et al., 2011, 2012). Such studies have improved the *S. mansoni* functional annotation, allowed for a deeper understanding of the genomic complexity and lineage-specific adaptations potentially related to the parasitic lifestyle, and pointed out several proteins as potential drug targets, including proteases.

Cysteine proteases, one of the four major classes of proteolytic enzymes, have been found in a wide range of taxonomic groups, from viruses to vertebrates. These peptidases are involved in many biological processes, such as catabolism, antigen processing, inflammation, dystrophy, and metastasis (Henskens et al., 1996). Protease inhibitors, such as cystatins, inhibit the enzymatic activity of cysteine proteases. Cystatins comprise a family of cysteine protease inhibitors identified in diverse taxonomic groups, including Platyhelminthes and Nematoda (Kordis and Turk, 2009). In humans, cystatins have evolved widely not only to regulate enzymes in pathways but also as a defense mechanism against proteases of invading pathogens (Toh et al., 2010).

In parasites, cystatins participate in normal physiological processes, but are also important pathogenicity factors, being directly involved in host-parasite interactions (Hartmann et al., 1997; Manoury et al., 2001; Schierack et al., 2003; Harnett, 2014).

Based on sequence similarity, the presence or lack of disulfide bonds, and physiological localization, cystatins were first classified in three families: family 1 (e.g., stefins), family 2 (e.g., cystatins), and family 3 (e.g., kininogens) (Barrett, 1986). Afterwards, in terms of number of cystatin domains and the presence of sequence features these proteins were classified into type 1, 2, and 3 (Rawlings and Barrett, 1990). In the present study we adopted the classification proposed by MEROPS database a resource for peptidases and protein inhibitors (Rawlings et al., 2014). The database uses a hierarchical structure-based classification in which each peptidase and inhibitor amino acid sequences are grouped into families based on statistically significant similarities. MEROPS classifies cystatin proteins as members of the I25 family, further subdivided into four subfamilies: I25A, I25B, I25C, and unclassified (**Figure 1**). This classification system is based on similarities between protein sequences and three dimensional structures. According to MEROPS classification, proteins containing a single inhibitor unit are termed simple inhibitor, and those containing multiple inhibitor units are termed as a compound inhibitor (Rawlings et al., 2014). However, several proteins containing cystatin domains cannot be easily included in a classification scheme, resulting in a number of cystatin family members that remain without classification in the subfamily level (Cornwall et al., 2003; Kordis and Turk, 2009; Siricoon et al., 2012).

One of the first cystatin proteins described in parasitic organisms was the onchocystatin (I25B), a highly antigenic protein encoded by the nematode *Onchocerca volvulus* (Lustigman et al., 1991, 1992). Onchocystatin was initially proposed to be involved in parasite protease regulation during the molting process in Nematoda. Afterwards, it was shown that this protein is also involved in modulation of host immune responses (Hartmann et al., 1997). The molecular interactions of parasite cystatins and host molecules have not yet been clearly determined, but it is believed that the mechanisms are similar to those demonstrated for other species (Klotz et al., 2011). Some examples of known host parasite interactions were previously described in nematodes in which I25B secreted cystatins inhibit host cathepsins such as B and H by *Haemonchus contortus* (Newlands et al., 2001), L and S by *Acanthocheilonema viteae* (Vray et al., 2002), and B and L by *Nippostrongylus brasiliensis* (Dainichi et al., 2001).

Although cystatin family members have been the subject of many studies in different organisms, little is known regarding functional diversification and evolution in *Schistosoma*. In this context, the information available for human homologs can be used in comparative studies, at sequence and structure level, in order to understand the interactions of *Schistosoma* cystatins and host cysteine proteases. The present study aimed to identify cystatin homologs on predicted proteomes of three *Schistosoma* species and other Platyhelminthes in order to have a landscape

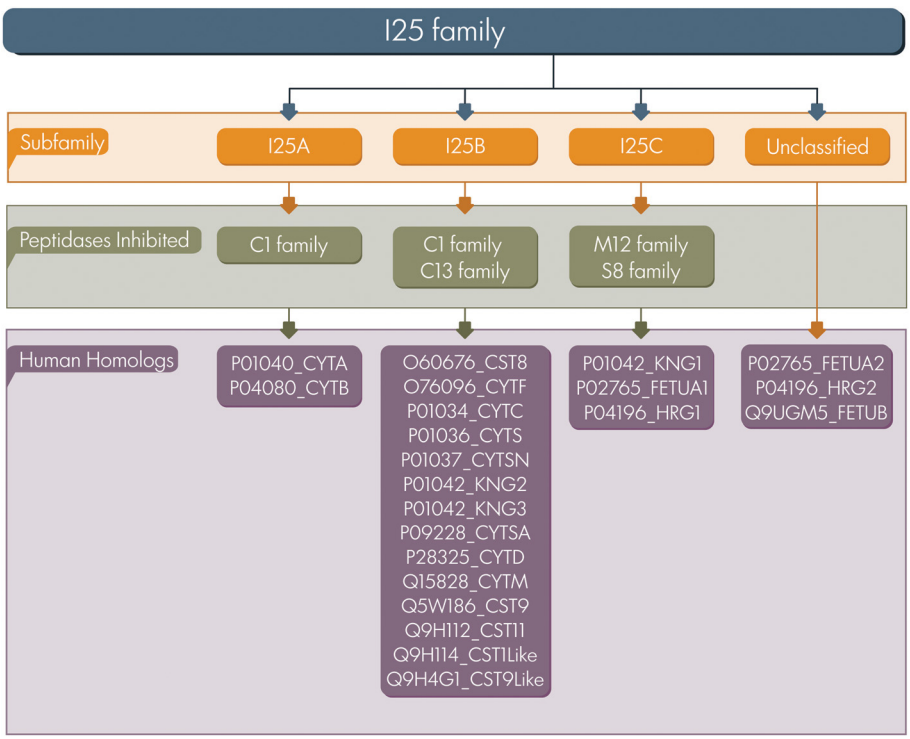

FIGURE 1 | Cystatin classification. The I25 family is classified in three subfamilies (I25A–C) according to MEROPS. Several family members remain unclassified. UniProt accession numbers of human homologs are listed.

view of the functional diversification in this phylum. In addition, evolutionary analyses were reconstructed for *Schistosoma* and human homologs based on the information at the sequence level, signatures, and phylogenetic relationships. Additionally, we evaluated cystatins' expression in different stages of the parasite life cycle in order to answer the following questions: How many cystatin homologs are present in *Schistosoma* species and in other Platyhelminthes? Do potential homologs have characteristic sequence features? What are the evolutionary relationships of the cystatin family members in *Schistosoma* species and their human homologs? Is the transcription of cystatin members during the *S. mansoni* life cycle stage-specific or is it conserved through the stages assessed?

In summary, we used predicted proteome data currently available for three *Schistosoma* species (Berriman et al., 2009; Zhou et al., 2009; Young et al., 2012), three Cestoda (Tsai et al., 2013), and the free living *Schmidtea mediterranea* (unpublished data) to identify potential cystatin homologs encoded by Platyhelminthes. Using combined computational approaches, we identified proteins belonging to the I25 family and reported members classified in two subfamilies: I25A and I25B. We also assessed microarray public datasets to investigate gene expression in different stages of the *Schistosoma mansoni* life cycle. This study provides insights into the evolution and potential functional diversification of Platyhelminthes cystatins improving our understanding of parasite biology and opening new frontiers in the identification of novel therapeutic targets against helminthiases.

MATERIALS AND METHODS

ORGANISMS AND SEQUENCE DATA

The dataset of selected species comprises three *Schistosoma* species: *S. haematobium* (NCBI taxid: 6185), *S. japonicum* (6182), and *S. mansoni* (6183); four other Platyhelminthes: *Echinococcus granulosus* (6210), *Hymenolepis microstoma* (85433), *Schmidtea mediterranea* (412041), and *Taenia solium* (6204); and *Homo sapiens* (9606). *Schistosoma* predicted proteomes were downloaded from SchistoDB 3.0 (beta.schistodb.net) (Zerlotini et al., 2013). Cestoda proteome data was obtained from the Sanger Institute FTP site (ftp.sanger.ac.uk/pub/pathogens). *S. mediterranea* proteome data was kindly provided by Dr. Eric Ross from Stowers Institute for Medical Research (USA). Predicted proteomes from each genome project were used in order to obtain evidence of protein gain or loss and a more accurate identification of cystatin homologs. *H. sapiens* I25 family members were retrieved from the Human Protein Reference Database (www.hprd.org) (Keshava Prasad et al., 2009). Functional information regarding the cystatin family is available on the MEROPS peptidase database (Rawlings et al., 2014) via the I25 inhibitor family identifier.

HOMOLOGS IDENTIFICATION

Potential cysteine protease inhibitors encoded by platyhelminth genomes were identified by using the hmmscan software included in the HMMER 3.0 package (Eddy, 2011). Each proteome was compared against Pfam-A HMM profiles, which were retrieved from the Pfam database (Finn et al., 2014). Such analyses were performed in order to identify the presence and architecture of proteins comprising the cystatin domain (Pfam: PF00031). The significance of the Pfam-A match is based on the resulting score. A match is considered significant when the score is greater than or equal to the gathering threshold for the Pfam domain. To date, the current threshold for the cystatin domain (Pfam: PF00031) is 20.9. Proteins containing significant or insignificant matches with the target domain were selected. Insignificant matches although less informative than significant ones, can be used for identifying functionally conserved regions when no significant matches are found. For this reason, insignificant matches were also initially selected in this work. In addition, information on accessory domains and protein signatures (Q-x-V-x-G motif, PW motif, LP motif, SND/SNS/TND motifs, and disulfide bonds) were considered to define potential I25 homologs. The presence of signal peptide in potential cysteine protease inhibitors was predicted by SignalP 4.1 using the neural network method with default D-cutoff values and using eukaryotes as "organism group" (Petersen et al., 2011). The illustrations of protein domain architectures were generated using DOG 2.0 (Ren et al., 2009).

PHYLOGENETIC ANALYSIS

Aiming at establishing evolutionary relationships among *Schistosoma* and experimentally validated human cystatins, I25A and I25B amino acid domain sequences from *S. haematobium*, *S. japonicum*, *S. mansoni*, and *H. sapiens* were selected for phylogenetic reconstruction. The evolutionary relationships between *Schistosoma* and human cystatins may provide cues about functions performed by parasites' orthologs. Human PF00031 domains classified into I25C subfamily or inhibitor units not assigned to a subfamily were not included in this analysis once they have no cysteine protease inhibitor activity. To optimize the dataset for phylogenetic analysis we removed redundancy and sequences too distantly related using the Decrease Redundancy tool, available as a resource at ExPaSy (www.expasy.org). The Decrease Redundancy parameters were set as 98 for "% max similarity" and 30 for "% min similarity." The filtered set of amino acid sequences, corresponding to the conserved domain (PF00031) were aligned using MAFFT 7 with iterative refinement by the G-INS-i strategy (Katoh et al., 2009). The multiple sequence alignment comprising 22 sequences and 96 sites was manually refined using Jalview (Waterhouse et al., 2009) and further used in phylogenetic analysis. To reconstruct the phylogenetic tree we used MrBayes 3.2.1, which performs Bayesian inference using a variant of the Markov Chain Monte Carlo (Ronquist and Huelsenbeck, 2003). MCMC analyses were run as four chains, one cold and three heated chains, for 10,000,000 generations and sampled every 100 generations. Twenty-five percentage of the initial samples were discarded as "burn-in." Mixed models were applied as a parameter to estimate the best-fit evolutionary model. Support values were estimated as Bayesian posterior probabilities. The evolutionary history of *Schistosoma* and human cystatins was also reconstructed based on the maximum likelihood method (ML), as implemented in PhyML (Guindon et al., 2010). For the phylogenetic reconstruction we tested 12 different evolutionary models (JTT, LG, DCMut, MtREV, MtMam, MtArt, Dayhoff, WAG, RtREV, CpREV, Blosum62, and VT) using the ProtTest 2.4 software (Abascal et al., 2005). The evolutionary model best

fitting the data (best fit model) was determined by comparing the likelihood of the tested models according to the Akaike Information Criterion. Trees were visualized and edited using the FigTree software (tree.bio.ed.ac.uk/software/figtree).

TRANSCRIPTIONAL PROFILES

Data from 35,437 oligonucleotide microarray probes from *S. mansoni* transcriptomic analyses (Fitzpatrick et al., 2009) were interrogated in order to identify the transcription patterns of two cystatin family members: Smp_006390 (I25A) and Smp_034420.2 (I25B). Thirteen development stages were covered and the complete set of raw and normalized data were downloaded from ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) under the experiment accession number E-MEXP-2094. For differential expression analysis, mean fluorescence normalized values were linear model fitted using three replicates per stage and a total of 19 evolutionary pairwise comparisons were made (see Fitzpatrick et al., 2009 for details). Additionally, recently published RNAseq transcription data (Protasio et al., 2012) was also interrogated for gene expression pattern and gene model evaluation. In this case, four developmental stages of *S. mansoni* were covered. Raw sequence datasets (three from cercariae stage, two from 3 h post-infection mechanically transformed schistosomula, two from 24 h post-infection schistosomulas, and one from adult worms), were downloaded from ArrayExpress under the accession number E-MTAB-451. The RNAseq reads were stored in a local server and aligned to the most recent version of the *S. mansoni* genome (v.5). Reads were mapped with Tophat-v.2.0.8 (Trapnell et al., 2012) and transcripts were assembled with Cufflinks-v.2.0.2 (Trapnell et al., 2012). Cuffdiff, a program from the Cufflinks suite, was used to estimate expression of transcripts across samples. CummeRbund, an R package, and Integrative Genomics Viewer -IGV (Thorvaldsdóttir et al., 2013) were used to visualize results.

RESULTS

In this study we have mined platyhelminth proteomes in order to identify proteins belonging to the I25 family and its respective subfamilies. To this end, we used intrinsic methods at sequence level followed by multiple sequence alignment and phylogenetic analysis. Such analyses generated an evolutionary view of potential cystatin proteins in three *Schistosoma* species. We also analyzed the amino acid sequence diversity focused on the identification of protein signatures. Finally, we verified the transcriptional profiles of cystatins. Overall, a framework for functional analysis of parasite cystatins is provided. In summary, our findings contribute to a better understanding of host-parasite interactions and pathogenesis, once analysis and cystatins appear as relevant molecules in these processes.

IDENTIFICATION OF CYSTATIN FAMILY MEMBERS

Cystatin family (I25) members were identified using an intrinsic method. Platyhelminth proteomes were scanned by hmmscan (Eddy, 2011) and potential homologs were retrieved based on the presence of significant or insignificant matches with the conserved cystatin domain (PF00031) (**Table 1**). In cases where insignificant PF00031 matches were recovered, we also searched for critical residues that mediate protease inhibition to define the query protein as a potential cysteine protease inhibitor (**Table 2**). Based on "start" and "end" alignment positions of potential homologs identified overlapping the PF00031 HMM profile, truncated regions were assigned. It is important to emphasize that the Pfam database (Finn et al., 2014) is built from the most recent UniProt (UniProt Consortium, 2014) release and that no single protein database covers all diversity existing in nature. More specifically, the total of platyhelminth cystatins available at UniProt is underrepresented when compared, for instance, to mammals. Thus, it is possible that the presence of divergent regions reflects their degree of divergence to other proteins available at the database. On the other hand, it is also important to consider that the difference between the PF00031 HMM profile and the query sequences can be related to the presence of pseudogenes or errors in the gene models.

Considering alternative splicing products (Smp_034420.1, Smp_034420.2, and Smp_034420.3; Sha_109477 and Sha_109478), we identified in *Schistosoma* species ten proteins that contain the conserved domain (**Table 1**). Three proteins were retrieved in *S. haematobium*, three in *S. japonicum*, and four in *S. mansoni*. These single domain proteins vary in length and in domain size. In order to classify the identified homologs in subfamilies (I25A–C or unclassified), we searched for the presence of signal peptide and other evolutionarily conserved residues (**Table 2**), which are involved in the formation of a wedge-like structure that is complementary to the active site of target proteases. Features as Q-x-V-x-G, LP, and PW motifs which are considered essential for binding and inhibiting cysteine proteases activity were identified. To remove potentially redundant sequences as well as too distantly related proteins we filtered alternative splicing products and run the Decrease Redundancy program using the previously mentioned parameters. In total, four sequences were filtered out: Sha_109478, Sjc_0094540, Smp_034420.1, and Smp_034420.3.

Concerning other Platyhelminthes species, we identified 14 cystatin proteins encoded by three Cestoda (*E. granulosus*, *H. microstoma*, and *T. solium*) and a free living Turbellaria (*S. mediterranea*) (**Table 1**). Contrary to *Schistosoma* species, hmmscan searches retrieved additional domains in some of those homologs. Such domains were retrieved as insignificant matches and a few showed overlapping regions with the conserved domain (PF00031) (**Table 1**). The information of additional domains may suggest potential lineage-specific innovations that happened in cystatin family members over evolutionary time. On the other hand, it can reflect the caveat of data quality in organisms for which we have only draft genomes.

We also analyzed cystatin diversity at the sequence level in terms of critical motifs, amino acid conservation, or variants that could lead to differences in the inhibitory capability of the cystatins (**Table 2** and **Figure 3**). The alignment of identified I25A and I25B cystatin sequences point out four conserved regions: a Glycine residue within the N-terminal region, a Q-x-V-x-G motif in one hairpin loop, and a PW or LP motifs in the second loop (**Figure 3**). Those regions can dock with the substrate-binding site of family C1 of cysteine proteases (Dickinson, 2002). One disulfide bridge exclusively present in I25B proteins was also identified.

Table 1 | Cystatin predictions across *Schistosoma* and other Platyhelminthes.

Taxon	TaxID	Accession	Length	Domain	Start	End	*E*-value	Score	Significant
Schistosoma haematobium	6185	Sha_109477	160	PF00031	83	142	2.2×10^{-1}	11.6	No
		Sha_109478	145	PF00031	38	127	4.5×10^{-2}	13.8	No
		Sha_300402	101	PF00031	35	91	1×10^{-6}	28.7	Yes
Schistosoma japonicum	6182	Sjc_0005780	145	PF00031	37	126	1.2×10^{-8}	35	Yes
		Sjc_0066340	101	PF00031	40	88	2.7×10^{-7}	30.6	Yes
		Sjc_0094540	123	PF00031	40	85	1.8×10^{-4}	21.5	Yes
Schistosoma mansoni	6183	Smp_006390.1	101	PF00031	35	91	1×10^{-5}	25.5	Yes
		Smp_034420.1	117	PF00031	38	98	8.3×10^{-7}	29	Yes
		Smp_034420.2	148	PF00031	38	129	7.9×10^{-8}	32.3	Yes
		Smp_034420.3	145	PF00031	38	126	1.5×10^{-2}	15.3	No
Echinococcus granulosus	6210	EgrG_000159200.1	98	PF00031	16	77	4.5×10^{-5}	23.5	Yes
		EgrG_000159200.1	98	PF03672	29	51	2.2×10^{-3}	17.5	No
		EgrG_000543900.1	111	PF00031	34	79	5.1×10^{-3}	16.9	No
		EgrG_000849600.1	274	PF00031	45	98	2.3×10^{-10}	40.4	Yes
		EgrG_000849600.1	274	PF00031	167	224	4.8×10^{-2}	13.7	No
		EgrG_000849600.1	274	PF13549	37	104	1.1×10^{-1}	11.7	No
Hymenolepis microstoma	85433	HmN_000582300.1	180	PF00031	41	83	2.4×10^{-2}	14.7	No
		HmN_000582400.1	107	PF00031	26	78	1.5×10^{-2}	15.4	No
		HmN_000582400.1	107	PF06050	18	51	4.4×10^{-2}	12.5	No
		HmN_000842000.1	295	PF00031	63	115	3.2×10^{-4}	20.7	No
Taenia solium	6204	TsM_000671000	274	PF00031	45	98	5.2×10^{-10}	39.3	Yes
		TsM_000671000	274	PF00031	175	224	1.7×10^{-2}	15.2	No
		TsM_000687900	98	PF00031	9	76	2.6×10^{-4}	21	Yes
		TsM_000687900	98	PF03672	29	51	2.2×10^{-2}	14.3	No
		TsM_000687900	98	PF13805	8	73	7.1×10^{-2}	12.2	No
		TsM_001154200	115	PF00031	37	84	4×10^{-3}	17.2	No
		TsM_001154200	115	PF14073	37	85	1.4×10^{-1}	11.9	No
		TsM_001154200	115	PF15606	39	87	3×10^{-2}	14.2	No
		TsM_001154300	137	PF00031	33	95	1.2×10^{-4}	22.1	Yes
Schmidtea mediterranea	412041	mk4.000249.00.01	93	PF00031	4	51	8.6×10^{-2}	12.9	No
		mk4.000249.04.01	93	PF00031	4	51	9.6×10^{-2}	12.8	No
		mk4.004385.02.01	119	PF00031	33	104	1.3×10^{-13}	50.8	Yes
		mk4.027397.00.01	176	PF00031	33	121	6.1×10^{-14}	51.9	Yes

TaxID: identifier at NCBI taxonomy database. Accession: accession number in the source genome project database. Length: number of amino acids. Domain: domain prediction based on HMM models from the Pfam database using the HMMscan tool. Start and End: alignment positions of the retrieved domain in the query sequence. Significant (Yes/No): statistically significant or insignificant score values according to the gathering threshold for each Pfam domain.

I25A subfamily members are predominantly intracellular single-domain proteins of about 11 kDa and ∼100 amino acid residues, which do not contain disulfide bridges and the PW motif. I25A inhibitors have three evolutionarily highly conserved regions: a glycine residue within the N-terminal region, a central Q-x-V-x-G motif, and a C-terminal LP pair (Klotz et al., 2011).

Figure 3 shows five *Schistosoma* and human homologs that have these conserved features, being therefore classified into the I25A subfamily. Following the same pattern of conserved features, when analyzing the proteome data of others Platyhelminthes (**Table 2**), we identified potential I25A subfamily member in *E. granulosus* (EgrG_000159200.1) and in *T. solium* (TsM_000687900). In *H. microstoma* three potential cystatin proteins without signal peptide were identified, something uncommon in other platyhelminth predictions. Therefore, this result should be further evaluated carefully, before being considered an evolutionary innovation. In *S. mediterranea* we identified two proteins belonging to the I25A subfamily. However the identified cystatins mk4.000249.04.01 and mk4.000249.00.01 are identical. For this reason we took into account the probability of redundancy and considered one of them (**Table 2**) as a cystatin homolog.

I25B inhibitors are secreted single-domain proteins around 14 kDa, ∼120 residues long with at least one disulfide bridge and

Table 2 | Sequence features of I25 family members in selected taxa.

TaxID	Accession	SP	SND/SNS/TND	Q-x-V-x-G	S-S	LP	PW
6185	Sha_109477	Yes	N/A	Yes	Yes	No	Yes
	Sha_109478	Yes	N/A	No	Yes	No	Yes
	Sha_300402	No	N/A	Yes	No	Yes	No
6182	Sjc_0005780	Yes	N/A	Yes	Yes	No	Yes
	Sjc_0066340	No	N/A	Yes	No	Yes	No
	Sjc_0094540	No	N/A	Yes	Yes	No	No
6183	Smp_006390.1	No	N/A	Yes	No	Yes	No
	Smp_034420.1	Yes	N/A	Yes	Yes	No	No
	Smp_034420.2	Yes	N/A	Yes	Yes	No	Yes
	Smp_034420.3	Yes	N/A	No	Yes	No	Yes
6210	EgrG_000159200.1	No	N/A	Yes	Yes	Yes	No
	EgrG_000543900.1	Yes	N/A	Yes	No	No	No
	EgrG_000849600.1_x	Yes	N/A	Yes	No	No	Yes
	EgrG_000849600.1_y	No	N/A	No	No	No	No
85433	HmN_000582300.1	No	N/A	Yes	No	No	No
	HmN_000582400.1	No	N/A	Yes	No	No	No
	HmN_000842000.1	No	N/A	Yes	No	No	No
6204	TsM_000671000_x	Yes	N/A	Yes	No	No	Yes
	TsM_000671000_y	No	N/A	No	No	No	No
	TsM_000687900	No	N/A	Yes	No	Yes	No
	TsM_001154200	Yes	N/A	No	No	No	No
	TsM_001154300	Yes	N/A	Yes	No	No	No
412041	mk4.000249.00.01	No	N/A	Yes	No	Yes	No
	mk4.000249.04.01	No	N/A	Yes	No	Yes	No
	mk4.004385.02.01	Yes	N/A	Yes	Yes	No	No
	mk4.027397.00.01	Yes	N/A	Yes	Yes	No	Yes
9606	O60676_CST8	Yes	No	No	Yes	No	Yes
	O76096_CYTF	Yes	Yes	Yes	Yes	No	Yes
	P01034_CYTC	Yes	Yes	Yes	Yes	No	Yes
	P01036_CYTS	Yes	No	No	Yes	No	Yes
	P01037_CYTSN	Yes	No	Yes	Yes	No	Yes
	P01040_CYTA	No	No	Yes	No	Yes	No
	P01042_KNG1	Yes	No	No	Yes	No	No
	P01042_KNG2	N/A	No	Yes	Yes	No	No
	P01042_KNG3	N/A	No	Yes	Yes	No	Yes
	P04080_CYTB	No	No	Yes	No	Yes	No
	P09228_CYTSA	Yes	No	Yes	Yes	No	Yes
	P28325_CYTD	Yes	No	Yes	Yes	No	Yes
	Q15828_CYTM	Yes	Yes	Yes	Yes	No	Yes
	Q5W186_CST9	Yes	No	No	Yes	No	No
	Q9H112_CST11	Yes	No	No	Yes	No	Yes
	Q9H114_CST1Like	Yes	Yes	No	Yes	No	Yes
	Q9H4G1_CST9Like	Yes	No	No	Yes	No	Yes

TaxID: identifier at NCBI taxonomy database. 9606: Homo sapiens. Accession: accession number in the source genome project database or in UniProt (human proteins). Sequence features include a signal peptide (SP), disulfide bridge (S-S), and distinct motifs (SND/SNS/TND, Q-x-V-x-G, LP, and PW), in which amino acids are indicated by the one-letter code. Yes or No: presence or absence of conserved features. N/A: Not applicable.

a signal peptide. I25B inhibitors have two of the three conserved regions previously mentioned: the N-terminal Gly residue and a central Q-x-V-x-G motif. Instead of a C-terminal LP pair, I25B inhibitors have a PW motif at the C-terminal segment. Besides, some I25B members also possess a distinct conserved SND, SNS or TND motifs between the first conserved glycine and the central Q-x-V-x-G motif (**Table 2**). The presence of these additional motifs allow cystatin proteins to inhibit either legumain or asparaginyl endopeptidases (Alvarez-Fernandez et al., 1999; Zavasnik-Bergant, 2008; Klotz et al., 2011; Schwarz et al., 2012). In parasites, I25B subfamily members were demonstrated to be involved in modulation of host immune responses (Khaznadji et al., 2005; Gregory and Maizels, 2008). **Figure 3** shows I25B *Schistosoma* and human sequences identified according the sequence features previously mentioned. In three Platyhelminthes species (*E. granulosus*, *H. microstoma*, *T. solium*) it was not possible to identify I25B homologs (**Table 2**). In *S. mediterranea* we identified two similar sequences. However, it seems like that mk4.004385.02.01 is a fragment of mk4.027397.00.01. In this case we have chosen the mk4.027397.00.01 protein as potentially true I25B homolog due to the presence of expected sequence features (**Table 2**).

I25C subfamily members act mostly on serine proteases classified into the family S8 (Cornwall et al., 2003) and metalloproteases from the family M12 (Valente et al., 2001). Fetuins and histidine rich proteins are also multi-domain secreted proteins, but lack cystatin activity and are called as unclassified (Rawlings et al., 2014). Kininogens and fetuins are much younger than I25A and I25B proteins and their occurrence are restricted to vertebrates (Kordis and Turk, 2009). According to our findings, the I25C and unclassified subfamily members are not encoded by the genomes of platyhelminth species here analyzed.

Human cystatin protein subunits previously characterized as protease inhibitors were classified as belonging to one of the three cystatin subfamilies 125A–C (**Table 2**). In total, 15 proteins were retrieved from the Human Protein Reference Database (Keshava Prasad et al., 2009) and evolutionarily conserved residues were identified (**Table 2** and **Figure 3**). As already described in the literature, one additional motif was found (SND/SNS/TND), which is related to legumain inhibition. A multidomain protein P01042 was retrieved for each separate subunit denoted as KNG1, KNG2, and KNG3 (**Table 2**).

EVOLUTIONARY RELATIONSHIPS AMONG *SCHISTOSOMA* AND *H. SAPIENS* CYSTATINS

The evolutionary relationships in cystatins were reconstructed from an alignment containing 22 sequences corresponding to the conserved domain (PF00031) and 96 sites (**Figure 3**) using maximum likelihood and Bayesian inference. Both methods retrieved the same tree topology. Statistical support was also calculated for each node by both phylogenetic inference methods (Bayesian inference/maximum likelihood). Protein sequences are represented on the phylogenetic tree by UniProt (UniProt Consortium, 2014) and SchistoDB (Zerlotini et al., 2013) identifiers. Based on the phylogeny we were able to identify two well supported monophyletic subfamilies (100/100): I25A and I25B (**Figure 4**). I25C and unclassified homologs were not identified in *Schistosoma* species. Two domains of the kininogen protein P01042 (KNG2 and KNG3) were placed in I25B clade due to its cysteine protease inhibitory activity. The KNG1 domain from the same protein belongs to the I25C subfamily and was not included in this analysis because it has lost its inhibitory activity due to mutations in structurally important regions (Kordis and Turk, 2009) and may act as a calcium transporter (Higashiyama et al., 1987).

According to the phylogeny (**Figure 4**), three *Schistosoma* proteins (Sha_300402, Sjp_0066340, and Smp_006390.1) and two human cystatin proteins (P04080_CYTB, P01040_CYTA) were grouped into the I25A subfamily clade. These proteins share the evolutionarily conserved residues: a N-terminal Gly, a central Q-x-V-x-G, and a C-terminal LP (**Table 2** and **Figure 3**). Based on the information available on the literature and in protein databases, both human homologs (P04080_CYTB, P01040_CYTA) were experimentally and structurally characterized. On the other hand, only two I25A *Schistosoma* proteins (Smp_006390.1 and Sjp_0066340) were experimentally characterized at the protein level (Morales et al., 2004; He et al., 2011). Those proteins are involved in a intracellular modulator role. For instance, Smp_006390.1 was able to inhibit the formation of hemozoin by live schistosomula, suggesting a possible role in the gut of the schistosomula (Morales et al., 2004).

In I25B subfamily clade three *Schistosoma* homologs were grouped with 13 human proteins (14 cystatin domains). The conserved features (signal peptide, Gly residue, Q-x-V-x-G motif, disulfide bridge, and PW motif) were not detected in all protein domain sequences (**Table 2** and **Figure 3**). Contrary to *Schistosoma* I25A subfamily members, I25B cystatins were not experimentally characterized. Human sequences placed on the I25B subfamily clade reveal a significant expansion of such subfamily in *H. sapiens*. The phylogenetic analysis shows that these homologs originated from successive post speciation gene duplication events. Most human I25B members present the typical protein signatures (**Table 2** and **Figure 3**).

In summary, *Schistosoma* cystatin clades of I25A and I25B subfamily members were well supported by both branch support methods showing 100/100 (aLRT and posterior probability). *S. haematobium* and *S. mansoni* cystatins are closest related to each other when compare to the *S. japonicum* homolog. The phylogenetic analysis showed that human and *Schistosoma* cystatins are placed in different clades, reflecting its diversity at molecular level.

CYSTATIN EXPRESSION IN *SCHISTOSOMA MANSONI*

According to the microarray data analysis (Fitzpatrick et al., 2009), both I25A and I25B members, Smp_006390.1 and Smp_034420.2 respectively, were constitutively expressed in several stages of the *S. mansoni* life cycle (**Figure 5**). These findings corroborate the pairwise comparisons of key developmental stages performed by Fitzpatrick et al. (2009) that did not indicate any differential expression of both cystatin mRNAs. Comparisons were made with an adjusted p value (Adj $p < 0.05$), corrected using the Benjamini and Hochberg method for multiple testing, for which 3 replicates per life cycle were assessed. Both cystatins expression were also confirmed in the RNA sequencing work of Protasio et al. (2012). Transcripts were expressed constitutively

in all four stages evaluated (data not shown). No differential expression assessment was considered in this case, mostly because those experiments did not include adult worm sample replicates. Therefore, without replicates, it is impossible to estimate sample variability.

As RNAseq data can be used to improve gene model annotations, current gene models for both I25A and I25B members were investigated. We analyzed the read coverage by mapping the reads against the reference genome. It was performed by Tophat, a tool that allows read alignments containing gaps in regions spanning introns. Therefore, predicted gene models for both cystatin family members were confirmed by visual inspection using IGV (Thorvaldsdóttir et al., 2013). All three exons of I25A and four exons of I25B, as well as both 5′ and 3′ UTR regions, located in the *S. mansoni* SuperContig 0138 and chromosome 2 respectively, had several reads correctly mapped within the exon limits (data not shown).

DISCUSSION

CONSERVED SEQUENCE FEATURES IN CYSTATINS

The I25A cystatin subfamily is a predominantly intracellular protein and does not present disulfide bridges. The inhibitor domain Q-x-V-x-G is present in the first hairpin loop and contains the glycine residue in the first position of the cystatin domain. This amino acid is also conserved in other cystatins of the I25B subfamily (**Figure 3**). This glycine residue allows the N-terminal region to interact with the sub-sites S4, S3, and S2 of the cysteine proteases. In *Schistosoma* species, besides its role in degradation of hemoglobin, I25A can also act intracellularly as a general regulator of protease activity (Morales et al., 2004). The constant and ubiquitous expression in *S. mansoni,* as shown by transcriptomic analysis, supports this idea. In the I25A subfamily we identified the highly conserved LP motif in positions 73–74 (**Figure 3**). The LP motif is essential for high affinity binding to papain (Pol and Bjork, 1999). The conserved motif differences between I25A and I25B subfamilies could reflect differences in the inhibitory spectrum of these proteins during evolution of function (Dickinson, 2002).

Kordis and Turk (2009) postulated that the progenitor of this family was most probably intracellular, lacked a signal peptide and disulfide bridges. The hypothesis is that throughout cystatins evolution, gene duplications combined with deletions and insertions of genetic material resulted in single and multidomain proteins with or without disulpfide bonds, glycosylated or not. Accordingly members of I25B subfamily likely evolved from I25A ancestors, which lack cysteine residues, acquiring disulfide bridges and signal peptide during evolutionary processes (Brown and Dziegielewska, 1997; Gregory and Maizels, 2008). In *Schistosoma* we identified a single I25B cystatin in each species, all of them containing critical motifs (**Table 2**). On the other hand, the *E. granulosus* protein EgrG_000849600.1 has signal peptide and two cystatin domains. One of them has an insignificant match with the PF00031 HMM profile (**Table 2** and **Figure 2**). This multidomain protein did not show disulfide bridges, which is not typical for secreted proteins. The *T. solium* protein TsM_000671000 displayed architecture very similar to *E. granulosus* (**Table 2** and **Figure 2**). Perhaps this indicates a protein architecture that is lineage-specific of Cestoda, although both of these species fall within the same cyclophyllidean family, and may not be representative of all tapeworms. The *H. microstoma* cystatins displayed an interesting protein architecture. They do not contain a signal peptide and also lack the LP motifs, unlike other I25A members (**Table 2**).

The legumain inhibitory motifs (SND/SNS/TND) are distinct from the papain binding motif (Q-x-V-x-G) (Alvarez-Fernandez et al., 1999). These sites are located on the opposite side of the papain binding site (Gregory and Maizels, 2008) and were present in four human sequences P01034_CYTC (SND), Q15828_CYTM (SNS), O76096_CYTF (TND), Q9H114_CST1Like (SND) in position 28 of the cystatin domain (**Table 2** and **Figure 3**). In *Schistosoma* and other Platyhelminthes, these motifs belonging to bifunctional cystatins were not present. However, in the nematode *Brugia malayi* the SND motif was identified in the secreted cystatin Bm-CPI-2 and was able to block the activity of mammalian legumain (Manoury et al., 2001). This inhibition profile is shared with other nematodes implying a dual function for nematode cystatins. In terms of adaptation to parasitism, Hartmann and Lucius (2003) compared I25B cystatins from filariae to those of *C. elegans*, and observed a distinct pattern of enzyme inhibitory activity and immunological properties.

Our results point to the diversity in terms of the presence and absence of sequence features used to classify cystatins (**Table 2**). An accurate cystatin classification based just upon these features is challenging. We used strict classification criteria, but we must take into account that many of the genomes here analyzed are still in their early versions and may contain inaccurate gene models, suggesting it is necessary to undertake manual curation for unambiguous annotation of cystatins and other proteins.

PHYLOGENETIC RELATIONSHIPS IN THE CYSTATIN FAMILY

We identified two cystatin subfamilies, I25A and I25B, in the *Schistosoma*-Human phylogeny (**Figure 4**). In this work, a comparative analysis of *Schistosoma* cystatins and other 15 experimentally validated human cystatins belonging to diverse subgroups provided insights into the abundance, diversity, and evolution of *Schistosoma* cystatin family members. According to our results, the I25 cystatin family has a few members in *Schistosoma*, including I25A and I25B subfamilies' representatives in each species. Human cystatins have diversified significantly during the course of evolution, both at the sequence and functional levels, indicating that the cystatin domain is a protein-protein interaction module that can interact with novel targets (Alvarez-Fernandez et al., 1999; Dickinson, 2002; Abrahamson et al., 2003; Cornwall and Hsia, 2003; Kordis and Turk, 2009).

A subgroup in the I25B subfamily, named Cres (cystatin-related epididymal spermatogenic)/Testatin, was identified in our study with statistical support 80/86. This clade comprises I25B *Schistosoma* sequences. In human, glycoproteins of the Cres/Testatin subgroup are expressed in reproductive tissues and their function may be related to reproduction (Frygelius et al., 2010). The topology of this subgroup showed in **Figure 4** is consistent with the phylogeny reported by Frygelius et al. (2010). Interestingly this subgroup lacks the consensus Q-x-V-x-G motif (**Table 2** and **Figure 3**). **Figure 4** shows that these proteins have a

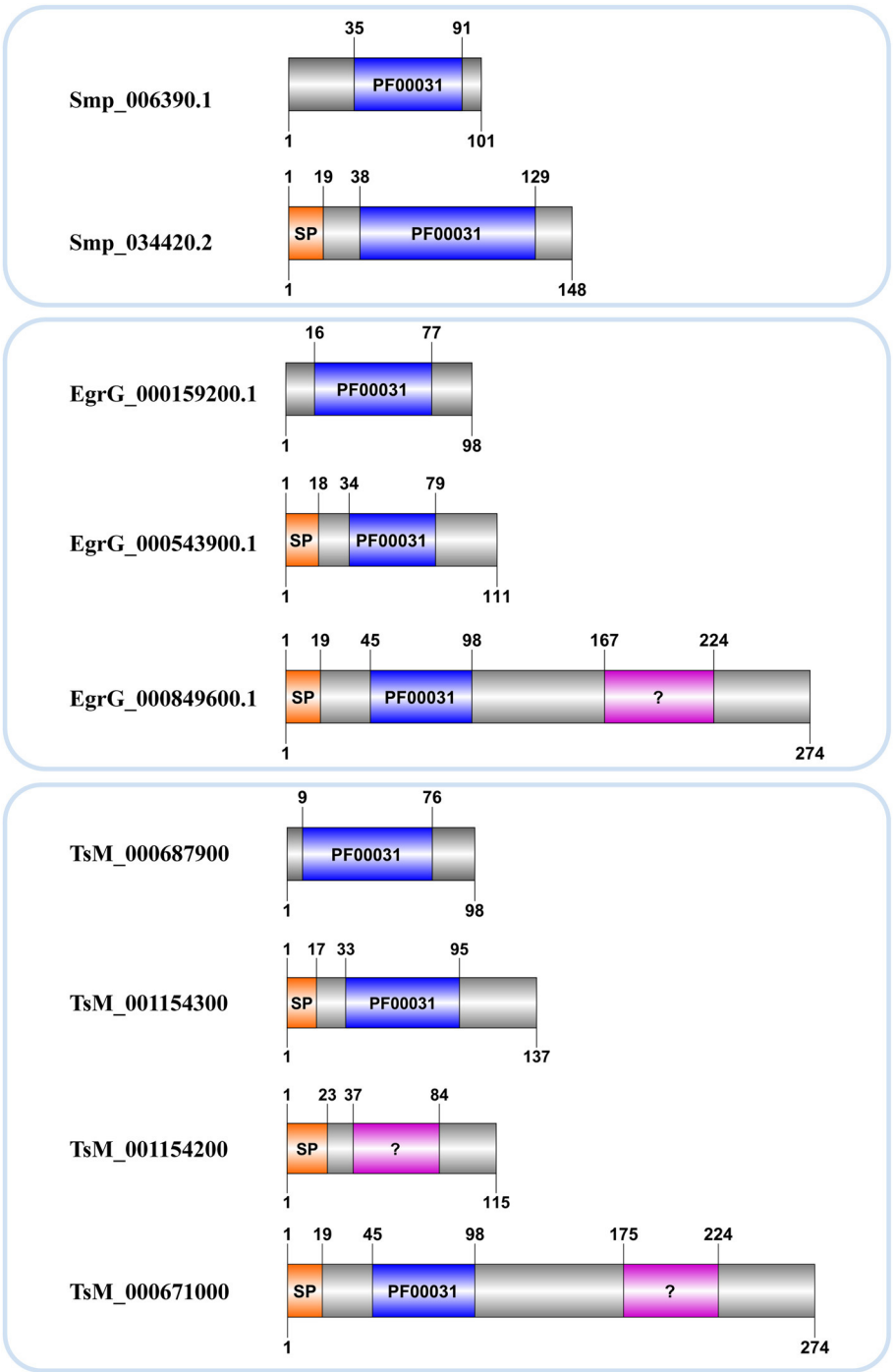

FIGURE 2 | Cystatin architecture in three Platyhelminthes. Accession numbers correspond to those assigned in each genome project. Domain limits (above) and sequence length (below) are provided for each protein. A signal peptide (SP) and the conserved domain (PF00031) with significant (blue) or insignificant (?) matches are indicated.

common origin and may represent a new subgroup within I25 family. Phylogenetic and comparative analyses show that genes involved in reproduction as Cres/Testatin and host pathogen interaction are under strong positive selection (Frygelius et al., 2010).

The phyletic distribution of the multidomain cystatins is limited and phylogenomic analyses suggest that multidomain cystatins are not monophyletic. Evidence suggests, they originated independently several times during evolution of eukaryotes (Kordis and Turk, 2009). Kininogen proteins (e.g., P01042) are

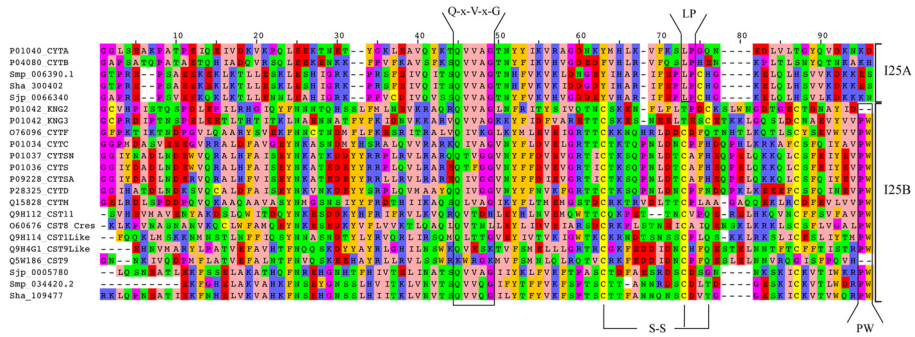

FIGURE 3 | Alignment of *Schistosoma* and human homologs. Multiple sequence alignment of the conserved domain (PF00031) of I25A and I25B proteins of *S. haematobium* (Sha), *S. japonicum* (Sjp), *S. mansoni* (Smp), and *Homo sapiens* (UniProt accession numbers). Amino acid sequences were aligned using MAFFT with iterative refinement by the G-INS-i strategy. Conserved motifs (Q-x-V-x-G and PW) and disulfide bridges (S-S) are indicated.

multidomain and divergent cystatins containing three domains with different inhibitory properties. In our phylogenetic analysis we discarded the first domain as it lacks inhibitory activity (Rawlings et al., 2014). The two remaining kininogen domains (P01042_KNG2; P01042_KNG3) were placed in the I25B subfamily clade. The P01042_KNG2 and P01042_KNG3 domains contain the Q-x-V-x-G residues critical for inhibitory activity (**Table 2**). Both domains are grouped together with other human cystatins Q15828_CYTM and O76096_CYTF that inhibit both cysteine and asparaginyl protease due to the presence of the SNS and TND motifs, respectively (**Table 2**).

Our results suggest that *Schistosoma* species contain only two cystatin subfamilies, reflecting little functional diversification. Due to the presence of highly divergent sequences in I25B clade, the recognition of orthologous sequences is a difficult task. The intracellular cystatins belonging to I25A subfamily are more conserved than the divergent extracellular cystatins (I25B) (**Figure 3**), as reported for other proteins with similar features (Julenius and Pedersen, 2006).

Khaznadji et al. (2005) reported the first I25A multidomain protein in invertebrates, a multidomain I25A in the platyhelminth *Fasciola hepatica* containing six cystatin like domains, two of which are well conserved (Khaznadji et al., 2005). The intracellular and multidomain I25A inhibits parasite cathepsin L1 activity. The methods used by Khaznadji et al. (2005) to determine the domain architecture of this cystatin differs from those applied by MEROPS (Rawlings et al., 2014), which indicates the presence of a single domain in this protein. In addition, the presence of multidomain proteins are not the only novelty in the cystatin family, several I25A cystatins from unicellular eukaryotic organisms have gained the signal peptide, which is absent in the majority of metazoan and eukaryotic I25A cystatins. The presence of signal peptide as observed in some unicellular eukaryotic I25A cystatins (Kordis and Turk, 2009) and in *Fasciola gigantica* (Siricoon et al., 2012) may lead to the gain of novel defense functions.

In synthesis, the major obstacle to the identification and classification of cystatins using amino acid sequences is the fact that many of the proteins contain multiple homologous inhibitor domains in a single protein (Rawlings et al., 2014). Furthermore, phylogenetic analysis of cystatin family members is hampered by short protein length often added to the sequence divergence. In addition, different branches appear to have evolved at different rates (Dickinson, 2002).

CYSTATIN EXPRESSION

In the present work, we interrogated publicly available gene expression datasets in order to investigate mRNA expression of both cystatin members I25A (Smp_006390.1) and I25B (Smp_034420.2) in *S. mansoni*. Microarray data by Fitzpatrick et al. (2009) assessed three ecological niches of *S. mansoni* life cycle (freshwater, molluscan, and definitive vertebrate host) and indicated similar expression levels of both cystatins among the parasite life cycle (**Figure 5**). The constitutive expression may be essential for schistosome life cycle progression. Published RNAseq data (Protasio et al., 2012) also point to the expression of cystatins in cercariae, schistosomula, and adults stages. Additional reports (Morales et al., 2004) suggest that I25A is expressed equally by adult males, females, and schistosomula stages. Therefore, both I25A and I25B are expressed throughout the *S. mansoni* life cycle.

In *S. japonicum*, He et al. (2011) observed not just the expression levels of the stefin Sjp_0066340, a I25A subfamily member, in egg, schistosomula, and adult stages by RT-PCR. He et al. (2011) also performed immunohistochemistry studies, which revealed that the *S. japonicum* stefin is mainly localized at the epithelial cells lining the gut as well as the tegument on the surface of adult worms. Additionally, the stefin of *Clonorchis sinensis* was also found mainly localized in the epithelial cells lining the intestine of the parasite (Kang et al., 2014). The stefin of *F. gigantica* was also localized in the intestinal epithelium and the tegumental type cell bodies together with the tegumental syncytium (Tarasuk et al., 2009). Altogether, the expression of parasite I25A proteins in the host-parasite interface point to a possible role in molecular interactions with host proteins, which are mostly inhibitors of host cysteine proteases such as cathepsins (Tarasuk et al., 2009; He et al., 2011; Kang et al., 2014).

Recently, an unusual secreted form of I25A member was characterized in *F. gigantica* (Siricoon et al., 2012). Although this awkward cystatin does present a signal peptide, typical of I25B proteins, sequence analysis does correlate it to the I25A subfamily. Nevertheless, Siricoon et al. (2012) provided evidence of a

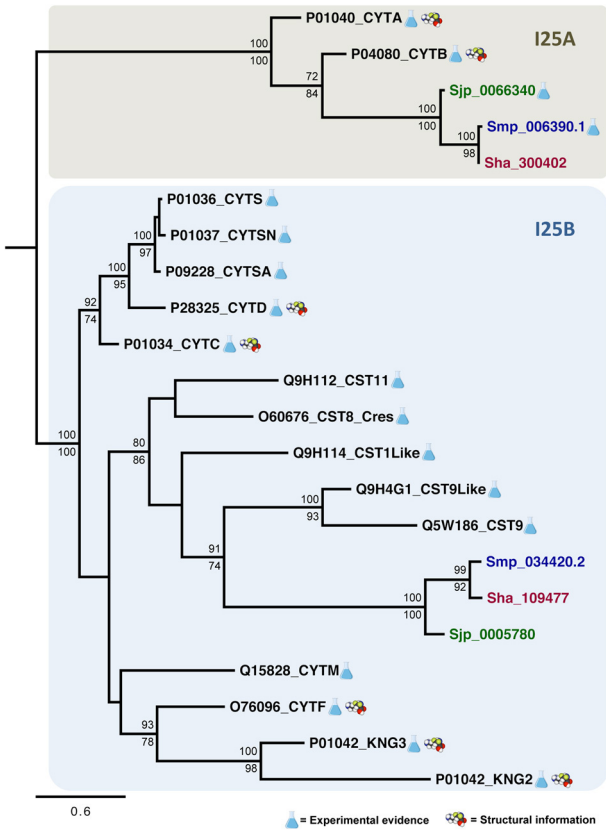

FIGURE 4 | Evolutionary relationships of I25A and I25B cysteine protease inhibitors. A total of 22 amino acid sequences and 96 sites comprising the conserved domain (PF00031) of homologs encoded by *S. haematobium* (brown), *S. japonicum* (green), *S. mansoni* (blue), and *Homo sapiens* (black) were analyzed. The phylogeny was reconstructed by two methods using WAG was as the best fit model. In the Bayesian inference, support values for each node were estimated as posterior probability (above). In the maximum likelihood analysis, they were estimated using the Akaike Likelihood Ratio Test (aLRT) (below). Only support values higher than 70% are shown.

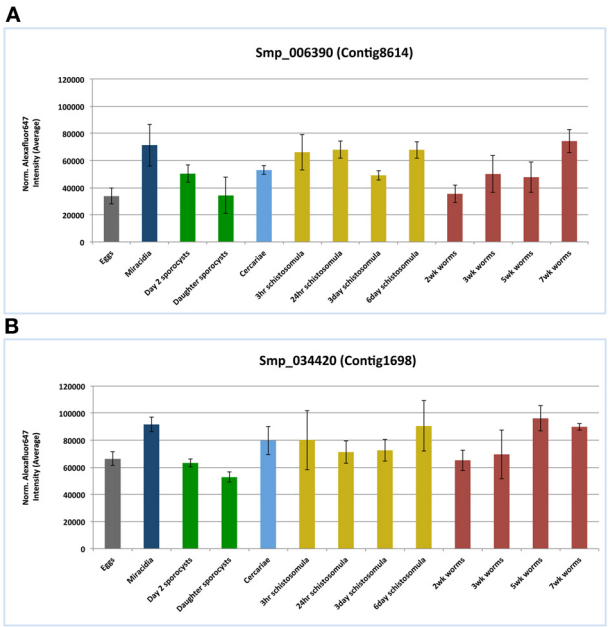

FIGURE 5 | Cystatins mRNA expression patterns in the *S. mansoni* life cycle. Public microarray data available at ArrayExpress (E-MEXP-2094) was downloaded to a local server in order to identify cystatins expression patterns of the I25A subfamily member Smp_006390 **(A)** and the I25B subfamily member Smp_034420 **(B)** in *S. mansoni*. Bars correspond to the mean normalized values for each oligonucleotide probe named Smp_006390 and Smp_034420 in 13 different life stages.

secreted form of a cystatin protein in Platyhelminthes that was again observed in the intestinal epithelium in all developmental stages. Moreover, the secreted 125A protein was also found expressed in the prostate gland in the adult stage of *F. gigantica*, which suggests a regulative role of cysteine protease activity in reproductive system. Similarly, the expression of human cystatin I25B subgroup proteins, also called Cres/Testatin, was localized at the reproductive tissues and their function may be related to reproduction (Frygelius et al., 2010). Interestingly, human Cres/Testatin subgroup was placed in the same clade with the *Schistosoma* cystatins I25B (**Figure 4**).

Based on the evidence of expression in related organisms and given the constitutive expression of cystatins I25A and I25B in *S. mansoni*, it is possible that cystatin functions can be involved in key processes in *Schistosoma*. Such proteins may be required to keep its proteolytic activity balanced as well as to protect the parasite against degradation by host or endogenous proteins. Nevertheless, cystatin tissue-specific expression, such as those identified at the reproductive system in human and *F. gigantica*, could evidence a more specialized role against specific cysteine proteases.

CONCLUSIONS

In summary, our evolutionary analysis using genomic, transcriptomic, and proteomic data for three *Schistosoma* species and other Platyhelminthes has provided the first insights into the evolution, classification, and functional diversification of platyhelminth cystatins. These findings improve our understanding concerning the diversity, at the molecular level, of cystatins encoded by such species. Only two subfamilies (I25A and I25B) were clearly identified in *Schistosoma* and Platyhelminthes reflecting the low diversification of this family when compared to human. Regarding Cestoda, it is necessary to implement an exhaustive study in order to better understand the domain composition revealed in our work.

We expect that this study will encourage experimental and structural characterization of cystatins in *Schistosoma* and other closely related parasites. Altogether, studies involving parasite cystatins will help to elucidate the functions performed by those proteins as well their correlation with parasite biology and host-parasite interaction. The importance of new insights revealed by functional genomics as RNAi experiments and comparative expression patterns across different life cycle stages in *Schistosoma* and other Platyhelminthes will provide a functional landscape of the cystatin role in the parasite life cycle.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Yesid Cuesta-Astroz, Larissa L. S. Scholte, Fabiano Sviatopolk-Mirsky Pais, Guilherme Oliveira, and Laila A. Nahum. Carried out homologs and protein signatures identification: Yesid Cuesta-Astroz and Larissa L. S. Scholte. Performed expression analysis: Fabiano Sviatopolk-Mirsky Pais and Yesid Cuesta-Astroz. Performed the phylogenetic studies: Yesid Cuesta-Astroz and Larissa L. S. Scholte. Wrote the manuscript: Yesid Cuesta-Astroz, Larissa L. S. Scholte, Fabiano Sviatopolk-Mirsky Pais, Guilherme Oliveira and Laila A. Nahum. Reviewed and revised the manuscript: Laila A. Nahum and Guilherme Oliveira. Coordinated this study: Laila A. Nahum and Guilherme Oliveira. All authors have read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health-NIH/Fogarty International Center (TW007012 to Guilherme Oliveira), the National Council for Research and Development, CNPq (CNPq Research Fellowship 309312/2012-4 to Guilherme Oliveira, INCT-DT 573839/2008-5 to Guilherme Oliveira, and CNPq-Universal 476036/2010-0 to Laila A. Nahum), and the Research Foundation of the State of Minas Gerais, FAPEMIG (CBB-1181/08 and PPM-00439-10 to Guilherme Oliveira). The authors are grateful to Dr. Eric Ross (Stowers Institute for Medical Research, USA) for providing the *Schmidtea mediterranea* proteome data. The authors acknowledge the use of the computing resources of the Center for Excellence in Bioinformatics (CEBio) and thank Mariana Aparecida de Oliveira Dias (CEBio) for the graphic design of **Figure 1**. Authors are very grateful to the two anonymous reviewers for their comments and suggestions to improve this work.

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Conflict of Interest Statement: The Guest Associate Editor Arnon Dias Jurberg declares that, despite being affiliated to the same institution as authors Cuesta-Astroz, Scholte, Pais, Oliveira and Nahum, the review process was handled objectively and no conflict of interest exists. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 April 2014; accepted: 18 June 2014; published online: 09 July 2014.
Citation: Cuesta-Astroz Y, Scholte LLS, Pais FS-M, Oliveira G and Nahum LA (2014) Evolutionary analysis of the cystatin family in three Schistosoma species. Front. Genet. 5:206. doi: 10.3389/fgene.2014.00206
This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Genetics.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 26 June 2014
doi: 10.3389/fmicb.2014.00313

Development of "-omics" research in *Schistosoma* spp. and -omics-based new diagnostic tools for schistosomiasis

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Schistosomiasis, caused by dioecious flatworms in the genus *Schistosoma*, is torturing people from many developing countries nowadays and frequently leads to severe morbidity and mortality of the patients. Praziquantel based chemotherapy and morbidity control for this disease adopted currently necessitate viable and efficient diagnostic technologies. Fortunately, those "-omics" researches, which rely on high-throughput experimental technologies to produce massive amounts of informative data, have substantially contributed to the exploitation and innovation of diagnostic tools of schistosomiasis. In its first section, this review provides a concise conclusion on the progresses pertaining to schistosomal "-omics" researches to date, followed by a comprehensive section on the diagnostic methods of schistosomiasis, especially those innovative ones based on the detection of antibodies, antigens, nucleic acids, and metabolites with a focus on those achievements inspired by "-omics" researches. Finally, suggestions about the design of future diagnostic tools of schistosomiasis are proposed, in order to better harness those data produced by "-omics" studies.

Keywords: *Schistosoma*, -omics, diagnosis, biomarkers, parasite

Schistosomiasis or "Bilharziasis" refers to the parasitic diseases caused by dioecious flatworms in the genus *Schistosoma*. The main pathogenic species of human schistosomiasis comprise *Schistosoma mansoni*, *S. japonicum*, and *S. haematobium*. Free living larvae of these pathogens released by variant kinds of snails in the fresh water, i.e., the cercariae can penetrate the skin of their definite hosts and subsequently bring about either egg-induced chronic responses, known as intestinal schistosomiasis (*S. mansoni and S. japonicum*) and urinary schistosomiasis (*S. haematobium*; Hu et al., 2004), or, less frequently, some acute reactions like toxemia or cytokine-induced shock (Wynn et al., 2004). These highly debilitating symptoms account for the severe morbidity and mortality incurred by schistosomiasis and render it a key poverty contributor for some developing countries in the tropical and subtropical regions. Moreover, schistosomiasis has also become an emerging threat in non-endemic areas due to the mounting numbers of immigrants and tourists (Patz et al., 2000; Enk et al., 2010). Currently, praziquantel (PZQ) based chemotherapy and morbidity control have become the predominantly adopted strategy for the treatment and control of schistosomiasis worldwide (Qing-Wu et al., 2002; Zhou et al., 2011b). Consequently, monitoring disease transmission, seeking patients for treatment in endemic areas and the evaluation of remedies entail viable and efficient diagnostic technologies, in order to optimize the extant control and prevention project for schistosomiasis (Balog et al., 2010).

STATUS QUO OF THE CURRENT SCHISTOSOMAL "-OMICS" RESEARCHES

"-omics" is a suffix labeling a series of biological subjects that adopt high-throughput experimental technologies to produce a large body of holistic and informative data. Since the launching of the *Schistosoma* Genome Project propelled by the WHO in 1994 (Oliveira et al., 2008), colossal progresses have been made in various *Schistosoma* -omics arenas including genomics, transcriptomics, proteomics, metabonomics (Ju et al., 2010), to name but a few. Besides an augmented understanding of schistosome biology and pathogenesis, those knowledge acquired by *Schistosoma* -omics researches also substantially contribute to the exploitation and innovation of drugs, vaccines as well as diagnostic tools for schistosomiasis. This review aims to display the representative achievements of those schistosomal -omics researches and conclude the diagnostic inventions derived from or at least inspired by those -omics studies.

TRANSCRIPOMICS

Schistosoma transcriptomics research commenced from the establishment of complementary DNA (cDNA) libraries which generated expressed sequence tags (ESTs) from nearly all stages of *S. mansoni* and *S. japonicum* (Fan et al., 1998; Fung et al., 2002; Hu et al., 2003; Peng et al., 2003; Shabaan et al., 2003; Verjovski-Almeida et al., 2003; Merrick et al., 2009). After the obtainment of numerous ESTs data, microarray and serial analysis of gene expression (SAGE) technology have been used to profile the transcripts of schistosomes in different stages and/or under distinct conditions. In particular, Geoffrey Gobert classified these transcriptomics applications into four main categories (Gobert, 2010), i.e., (a) characterizing individual cell/tissue types, (Jones et al., 2007; Gobert et al., 2009a), (b) profiling the intact organism and lifecycle (Hoffmann et al., 2002; Fitzpatrick et al., 2004, 2005, 2009; Hoffmann and Fitzpatrick, 2004;

Chai et al., 2006; Dillon et al., 2006, 2008; Gobert et al., 2006, 2009b; Moertel et al., 2006; Vermeire et al., 2006; Jolly et al., 2007; Ojopi et al., 2007; Williams et al., 2007; Hu et al., 2009; Taft et al., 2009), (c) parasite–host interactions and effect of therapies on parasite (Hoffmann et al., 2001; Alger and Williams, 2002; Sandler et al., 2003; Aragon et al., 2008, 2009; Gobert and Jones, 2008; de Moraes Mourão et al., 2009; You et al., 2009; Burke et al., 2010; Gobert et al., 2010), and (d) gene expression differences between geographical isolates or species (Le et al., 2002; Fitzpatrick et al., 2004; Moertel et al., 2006). Not until 2012 was the transcriptome of the adult and egg stages of *S. haematobium* profiled along with its genome (Young et al., 2012).

MicroRNA (miRNA) GENOMICS

Unlike messenger RNAs (mRNAs), small non-coding RNAs (sncRNAs) represent a group of untranslatable transcripts which are approximately 18–30 nt in length and serve as critical regulators to silence or activate specific target genes in a variety of organisms (Bartel, 2004; Molnar et al., 2010). Endogenous-small interfering (Endo-siRNAs), miRNAs, and Piwi-interacting RNA (piRNAs) are three main components of sncRNAs (Kim, 2005). Using protocols similar to the conventional transcriptomic researches, which can be outlined as RNA isolation, library construction, and sequencing (Cheng and Jin, 2012), vast numbers of schistosomal miRNAs and endo-siRNAs have been successfully detected in *S. mansoni* (Copeland et al., 2009; de Souza Gomes et al., 2011; Simões et al., 2011) and *S. japonicum* (Xue et al., 2008; Copeland et al., 2009; Hao et al., 2010; Wang et al., 2010b; Cai et al., 2011). What's more, recently Cai et al. (2012) adopted a totally novel method, i.e., the immunoprecipitation of *Sj*Ago2, a key factor in sncRNAs biogenesis with monoclonal antibodies (mAbs) to identify and characterize the associated small RNAs. Further classification steps showed that endo-siRNAs derived from transposable elements (TEs) were prominent among those conjugated sncRNAs.

PROTEOMICS

Since the correlation between transcriptional level and translational level of one expressed gene is not necessarily straightforward, proteomic analyses are also of great importance aiming at the characterization and comparison of functions, abundance, and subcellular localization (Ju et al., 2010) of numerous gene products derived from single or multiple samples. In a typical proteomics research, schistosomal specimen would be separated by one dimensional-(1D-) or 2D-polyacrylamide gel electrophoresis (PAGE) in the beginning. Subsequently, the isolated bands or spots would be subjected to digestion and either Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to the separation method. Finally, protein identities within the sample would be acquired through comparing the mass spectrometry (MS) results to the theoretical masses stocked in a particular database (van Hellemond et al., 2007). Based on the purpose of proteomics researches, schistosomal samples ought to be prepared in different ways. So far, schistosomal proteomics has been applied to the investigation and comparison of protein compositions in various developmental stages (Curwen et al., 2004; Liu et al., 2006) or between different genders (Cheng et al., 2005; Liu et al., 2006) and the worm proteins might be prefractioned accordingly, e.g., soluble membrane protein (Curwen et al., 2004; Cheng et al., 2005), tegumental fractions (Van Balkom et al., 2005; Braschi et al., 2006; Braschi and Wilson, 2006; Liu et al., 2006; Mulvenna et al., 2010; Castro-Borges et al., 2011), secreted antigens (Knudsen et al., 2005; Curwen et al., 2006; Liu et al., 2009), gut contents (Delcroix et al., 2007), etc.

IMMUNOMICS

Immunomics is a study that combines proteomics with serology and aims at ascertaining the interaction between host immune system and exotic antigens after pathogen invasion. Early schistosomal immunomics work utilized combined 2D Western blotting and MS to identify and compare proteins recognized by serum samples from *S. haematobium* exposed patients before and after PZQ treatment (Mutapi et al., 2005) or with different ages and infection intensities (Mutapi et al., 2008). Afterwards, an immunomics protein microarray technology was also applied in an attempt to seek prospective vaccine targets (Driguez et al., 2010). This advanced technology consists of several steps including genes selection, cell-free expression, chip printing, and serological probing.

GLYCOMICS

In view of the critical role glycans played in the induction of innate immune responses during schistosome–host interplay, glycomics, the profiling of the schistosomal protein- and lipid-conjugated glycan or glycan elements was addressed even before the screening of schistosomal transcriptomics and proteomics (Hokke et al., 2007). So far, the patterns of glycoconjugates expressed by multiple life stages of *S. mansoni* have been precisely elucidated by either anticarbohydrate mAbs identification or MS-based method (Khoo et al., 1995, 1997a,b, 2001; van Remoortere et al., 2000, 2003; Wuhrer et al., 2000, 2002, 2006a,b; Huang et al., 2001; Nyame et al., 2003; Robijn et al., 2005, 2007a,b).

GENOMICS

Under the continual efforts from two consortia of researchers, the genome of *S. mansoni* and *S. japonicum* were deciphered and published simultaneously in 2009 (Berriman et al., 2009; The *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium, 2009). Three years later, the genome information of *S. haematobium*, the pathogen causing urogenital schistosomiasis also became accessible (Young et al., 2012). Overall, the genome of the preceding three main causative human schistosomiasis pathogens are organized into eight chromosomes, including seven pairs of autosomes and a pair of ZW type sex chromosomes. The genome of *S. mansoni* (363 Mb), *S. japonicum* (397 Mb), and *S. haematobium* (385 Mb) encompass 13,184, 13,469, and 13,073 protein-coding genes, respectively, and a considerable proportion of them were mapped into gene ontology (GO) categories. Apart form those protein coding genes, more than 40% of these three genomes are taken up by repetitive elements, which contains retrotransposons, satellites, DNA

transposons and other kinds of unknown repeats. As a whole, the relationship between *S. mansoni* and *S. haematobium* are more intimate than either of them with *S. japonicum* (Young et al., 2012).

METABONOMICS

Schistosomes-related metabonomics keeps tabs on the overall changes of metabolites within tissues and biofluids of the hosts before and after blood fluke infection. Metabonomics-based schistosomiasis biomarker discoveries were pioneered in 2004 by Wang et al. (2004), who used ^{1}H nuclear magnetic resonance (NMR) spectroscopy and multivariate pattern recognition to analyze the metabolic signature of urine samples from *S. mansoni* infected mice. Subsequently, using various spectroscopic methods, e.g., NMR spectroscopy, MS, and capillary electrophoresis (CE), coupled with various data-mining technologies (Legido-Quigley, 2010), several metabonomics related to schistosome infection were profiled, such as urine samples from mice infected with *S. mansoni* (Garcia-Perez et al., 2008, 2009, 2010) and hamsters infected with *S. japonicum* (Wang et al., 2006), as well as blood and multiple tissue samples from schistosome-alone infected (Li et al., 2009) or two pathogens co-infected rodents (Wu et al., 2010). It was not until early this decade that one metabonomic investigation started to survey human *Schistosoma* spp. infection (Balog et al., 2011). Corresponding works will be reviewed in the next section.

THE "NEW GENERATION" DIAGNOSTIC METHODS FOR SCHISTOSOMIASIS AND THEIR PROGRESSES IMPELLED BY THE "-OMICS" ACHIEVEMENTS

TRADITIONAL, ETIOLOGICAL DIAGNOSTIC METHODS

Etiological diagnosis of schistosomiasis, i.e., the microscopic detection of schistosome eggs in urine (*S. haematobium*) or stool (*S. mansoni* and *S. japonicum*) samples is the most straightforward and widely adopted diagnostic approach to investigate the infection of schistosomes (Utzinger et al., 2011). Representative methods of etiological diagnosis include Kato–Katz (KK) thick smear (Katz et al., 1972), miracidium hatching assay (Jurberg et al., 2008), egg floating technique (FLOTAC; Cringoli, 2006), nuclepore urine filtration (Mott et al., 1982), etc. Those etiological methods are deemed as the "gold standard" for the detection of cases particularly in high endemicity settings and in the early stage of a control program. However, after mass drug administration or in low transmission settings, these traditional methods show limited sensitivity and accuracy (Zhao et al., 2012). In addition, multiple stool examinations or combined techniques, although reduced the false-negative rate to some extent, are quite time-consuming and labor-intensive, which hindered their application in large-scale epidemiological surveys (Zhou et al., 2011b). Thus, they have been gradually replaced by immunological techniques based on antigen or antibody detection, as well as PCR-based molecular tools, metabolic profiling, i.e., those so-called "new generation" diagnostic methods. Meanwhile, those novel diagnostic methods have benefited continuously from the previously introduced "-omics" researches (**Table 1**).

ANTIBODY-BASED, INDIRECT IMMUNOLOGICAL TECHNIQUES

Indirect immunodiagnostic assays use a range of immunological methods, such as circumoval precipitin test (COPT), indirect hemagglutination assay (IHA), dipstick dye immunoassay (DDIA), and enzyme-linked immonosorbent assay (ELISA; Zhu, 2005), etc. in order to detect schistosome-specific antibodies. Immunodiagnosis based on the detection of antibodies is relatively repeatable, sensitive, tractable, and time-saving compared with traditional etiological methods. Early indirect immunodiagnosis mainly relied on the crude extracts of worm components, like microsomal extract, gut-associated polysaccharide, non-fractionated extracts of eggs, etc. (Doenhoff et al., 2004) as diagnostic antigens. However, the usage of crude antigens frequently poses a cross-reaction problem because of those components within the crude antigens shared with other, irrelevant pathogens (Jin et al., 2010). Besides, antibody-based serological assays are not quantitative and usually fail to discriminate between previous exposure and current infection. Although it is difficult to eradicate all of these innate drawbacks of these diagnostic techniques, they could be alleviated to some extent by the use of selected recombinant antigens (Doenhoff et al., 2004). Thanks to the enormous amount of gene sequence information procured by the transcriptomics and genomics researches, together with those screening methods including proteomics, immunomics as well as some bioinformatics tools, many promising diagnostic antigens have been identified and their immunogenicity have been testified, vastly enriching the diagnostic antigen pools for schistosomiasis.

Complementary DNA libraries represent a sort of precious transcriptomic resources. Under combined efforts of serological screening and immunological verification, schistosomal antigens with high immunogenicity could be identified in a cDNA library and finally applied to antibody-based immunodiagnosis of schistosomiasis. Zhou et al. (2010) used rabbit sera collected on day 21 post-infection in an antigenic screening of a previously constructed cercariae cDNA library of *S. japonicum* (Fung et al., 2002). From the identified positive clones, the authors finally selected Sjp40 as an antigen candidate and used time-resolved fluoroimmunoassay (TRFIA) to profile the levels of anti-Sjp40 IgG in the sera of rabbits with different days post-infection. The result showed that compared with the controls at each time interval, since 21 days post-infection, the titer of circulatory anti-Sjp40 IgG in the infected groups started to significantly increase. Thus, it was considered that Sjp40 was a potential antigen for early diagnosis of schistosomiasis.

Likewise, another group of researchers (Zhou et al., 2009) used human saliva, instead of rabbit sera, to screen a cDNA library of *S. japonicum* eggs, which resulted in the detection of Sj13. The following ELISA assay exhibited 92.50% sensitivity and 92.11% specificity of salivary IgG detection with recombinant Sj13. Given the easy-accessibility and non-invasiveness of the saliva samples, saliva/Sj13 was asserted as a promising alternative to serological test for schistosomiasis. In addition to these two examples, numerous antigens, sorted from the cDNA library established in 2003 (Hu et al., 2003), like *Sj*EFCAB (Lu et al., 2012a), *Sj*E16 (Wang et al., 2003), *Sj* myophilin-like protein (Peng et al., 2008), P7 antigen (Xu et al., 2011), etc. have been cloned and expressed

Table 1 | An overview of "-omics" researches' contribution to the revelation of novel schistosomiasis diagnostic targets.

Diagnostic techniques	"-omics" researches		Representative diagnostic targets
Antibody-based immunodiagnosis	Transcriptomics	Fung et al. (2002)	Sjp40 (Zhou et al., 2010)
		Wu et al. (1998)	Sj13 (Zhou et al., 2009)
		Hu et al. (2003)	*Sj*EFCAB (Lu et al., 2012a), SjE16 (Wang et al., 2003), *Sj* myophilin-like protein (Peng et al., 2008), P7 antigen (Xu et al., 2011), 14-3-3 (Zheng et al., 2007), etc.
	Genomics	Berriman et al. (2009)	Sm200, Sm12.8, Sm43.5, Sm127.9, Sm18.9, Sm16.5 (Carvalho et al., 2011)
		The *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium (2009)	Sj1TR, Sj4TR, Sj7TR (Angeles et al., 2011)
	Immunomics	Zhong et al. (2010)	*Sj*LAP and *Sj*FBPA (Zhong et al., 2010)
Antigen-based immunodiagnosis	Immunomics	Lu et al. (2012b)	protein BUD31 homolog, ribonuclease, SJCHGC06971 protein and SJCHGC04754 protein
PCR-based molecular method	Genomics	The *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium (2009)	SjCHGCS19 (Guo et al., 2012)
Metabolic biomarkers discovery	Metabonomics	Balog et al. (2011)	Dimethylamine, Hippurate, PAG, etc. (Balog et al., 2011)

in *E. coli* prokaryotic expression system even earlier and their immunogenicity and diagnostic potential were also investigated. Despite of their considerable sensitivity and specificity as diagnostic antigens, since most of them were published in Chinese journals, their significance thereby waned.

Besides cDNA libraries, schistosomal genomes stand for another kind of resources to search potential indirect diagnostic antigens for schistosomiasis. Using bioinformatics screening, another group succeeded in identifying several novel diagnostic antigen candidates from the previously sequenced *S. mansoni* genome (Berriman et al., 2009). Overall, four criteria were used in the *in silico* analysis strategy, including expression in all parasite life stage within the definitive host, extracellular or plasmatic membrane localization, low similarity to human and other helminthic proteins and presence of predicted B cell epitopes (Carvalho et al., 2011). As a result, six promising diagnostic antigens (Sm200, Sm12.8, Sm43.5, Sm127.9, Sm18.9, Sm16.5) were elicited from 13,197 transcripts described for *S. mansoni* (Berriman et al., 2009). Ensuing verification steps including 1D- and 2D-Western blotting using schistosomula antigen preparation, adult worm preparation and sera from infected mice indicated the good immunogenicity of those predicted antigens.

Furthermore, bioinformatics techniques also served as screening tools to seek antigen candidates for indirect immunological diagnosis in the *S. japonicum* genome (Angeles et al., 2011). A group of antigens termed tandem repeat (TR) proteins were targeted in this study because of their role in humoral responses known previously (Kim et al., 2001; Goto et al., 2010). Specifically speaking, TR genes were identified from *S. japonicum* genome by a program named Tandem Repeats Finder. A total of 12,657 gene sequences were analyzed and 134 genes were found to have TR regions. Four TR genes, i.e., *Sj*1TR, *Sj*2TR, *Sj*4TR, and *Sj*7TR were selected for further research from the top 20 hits elicited by *in silico* screening based on their biochemical properties, conservation with other organisms, expression evidence, etc. Their recombinant proteins in expected size were successfully expressed and purified and ELISA was performed using sera from healthy people in endemic or non-endemic countries, post-treated individuals, stool-confirmed patients and patients of other parasitic diseases. *Sj*7TR had the highest sensitivity (85.71%), while both *Sj*1TR and *Sj*7TR had 100% specificity. More importantly, no cross-reaction with sera from patients infected by other pathogens was detected among those TR proteins. In contrast, the crude antigen of *S. japonicum*, SEA, had a higher sensitivity of 97.14% compared with the recombinant antigens but its specificity was poor (71.76%) and cross-reaction emerged with *Paragonimus westermani*, *Opisthorchis viverrini*, and *Entamoeba histolytica*-positive samples. In conclusion, *Sj*7TR was a promising candidate antigen for diagnosis purpose found by this research.

In another study, immunomics methods were also employed to uncover prospective diagnosis antigens for schistosomiasis (Zhong et al., 2010). In the beginning, more than 30 immunodominant spots were recognized by pooled sera from infected rabbits with Western blotting, 10 of which were precisely matched to the

homologous 2D-gel. LC/MS-MS was then adopted to identify those 10 spots and they were found to correspond to four distinct proteins. Two of the four identified proteins that had not been investigated before, namely, *Sj*LAP, a complex of metallopeptidases and *Sj*FBPA, a central glycolytic enzyme were successfully cloned and the recombinant protein products were further applied in the diagnosis of *Schistosomiasis japonica* by ELISA, which yielded sensitivities of 98.1 and 87.8% for acute and chronic schistosomiasis with r*Sj*LAP and 100 and 84.7% with r*Sj*FBPA, respectively, whereas the specificities were 96.7% for both antigens. Moreover, both antigen ELISA assays showed more than 80% sensitivity in diagnosis of chronic schistosomiasis with a low intensity infection and significantly declined antibody titers after the treatment with PZQ. Thus, r*Sj*LAP and r*Sj*FBPA proved to be useful diagnostic antigens for *S. japonicum* infection.

ANTIGEN-BASED DIRECT IMMUNOLOGICAL DIAGNOSIS

Early immunoassays have shown that schistosome-derived antigens, such as adult worm antigen (AWA), soluble egg antigen (SEA), and circulating antigen (CA; Zhao et al., 2012) could be released into the host circulatory system/excreta by schistosomes, which facilitates the researches on the direct immunological diagnosis of schistosomiasis. Circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) are by far the most extensively used antigens for the antigen-based immunodiagnosis of schistosomiasis and methods for the detection of CAs encompass sandwich ELISA (De Jonge et al., 1988), IHA (Deelder et al., 1989), time-resolved immunofluorometric assay (TR-IFMA; De Jonge et al., 1989), magnetic bead immunoassay (Gundersen et al., 1992), reagent strips (Van Etten et al., 1994), and liquid-phase piezoelectric immunosensor (LP-PEIS; Wen et al., 2011). Compared with the two foregoing diagnostic methods, the detection of CA is more suitable for drug efficacy trials owing to its high specificity and the ability to discriminate between previous exposure and current infection. Moreover, CAA and CCA can be readily detected in urine (Van Dam et al., 2004), which is easier and less invasive to be collected than blood required for antibody detection. Nonetheless, low sensitivity (Van Lieshout et al., 1995, 2000), failing to distinguish different species (Agnew et al., 1995) are disadvantages associated with this method.

Research conducted by Lu et al. (2012b) becomes the first and currently the sole immunomics-based study in an attempt to profile CAs in *Schistosoma* spp. In this study, AWA was initially prepared using *S. japonicum* worms collected from infected rabbits and then employed to subcutaneously immunize the hyline hens so as to produce anti-AWA IgY. The IgY antibody contained in the egg yolk was purified by water-dilution and ammonium sulfate precipitation approach and characterized by ELISA and Western blotting. Afterwards, purified IgY was immobilized onto aldehyde-activated beaded agarose resin and served as a capture antibody to immunoprecipitate and enrich CAs within the sera of patients. At last, the obtained CAs were separated by 1D electrophoresis and analyzed by LC-MS/MS, which lead to the identification of four proteins, i.e., protein BUD31 homolog, ribonuclease, SJCHGC06971 protein, and SJCHGC04754 protein. The following analysis indicated that those four CAs belonged to neither CAA nor CCA and had not been reported from previous proteomics researches. CAs revealed by corresponding -omics researches, just like these four, ought to be cloned and expressed in the future and the produced mAbs could be applied for antigen detection through sandwich ELISA which stands for a promising way to overcome the shortcomings of the current direct immunodiagnostic methods.

In addition to this finished work, one recent review about proteomics at the schistosome–mammalian host interface (Wilson, 2012) also proposed several candidate antigens that can be potentially used for CA detection, including serpin, α2 macroglobulin, Am200, LMWP, as well as some MEG-2 and MEG-3 proteins secreted by eggs.

NUCLEIC ACID AMPLIFICATION-BASED MOLECULAR METHODS

Given the existence of schistosomal DNA in the serum and other tissue samples of the host derived from dead worms, tegument shedding or inactive eggs (Xu et al., 2013), PCR-based molecular diagnosis has become a promising alternative to overcome the innate shortcomings of etiological and immunological diagnosis for schistosomiasis. Since the publication of the proof-of-concept study in 2002 (Pontes et al., 2002), PCR-based molecular diagnosis has been applied to the detection of schistosomes in multiple diagnostic field works (Pontes et al., 2003; Obeng et al., 2008; Enk et al., 2012) and several novel technologies, such as real time PCR (RT-PCR; Lier et al., 2006), PCR-ELISA (Gomes et al., 2010), and loop-mediated isothermal amplification (LAMP; Xu et al., 2010), etc. have been added to the tool assemblies in addition to conventional PCR in order to boost the detection sensitivity. Empirical evidence showed that molecular diagnosis of schistosomiasis has both high sensitivity and high specificity, enables detection during larval stage or at least before egg spawning and can use manifold materials, e.g., feces, serum (Pontes et al., 2002), plasma (Wichmann et al., 2009), and urine (Sandoval et al., 2006) as templates, all of which make it superior to the conventional diagnostic approaches. Factors that impact the result of molecular diagnosis are various and the choice of amplified products is definitely among them. However, confined by the inaccessibility to the schistosomal genome sequence, in the pre-genomic era, researchers could only select targets for PCR reactions within limited candidates. Representative examples include two different 121-bp repetitive elements in *S. mansoni* (Hamburger et al., 1991) or *S. haematobium* (Hamburger et al., 2001), *S. japonicum* 18s rDNA (Zhou et al., 2011a), highly repetitive retrotransposon *Sj*R2 (Laha et al., 2002), mitochondrial NADH1 gene (*nad1*) of *S. mansoni* (Jannotti-Passos et al., 1997) and *S. japonicum* (Lier et al., 2008), etc.

As early as 2009, 25 novel retrotransposons had been identified along with the decoding of the genome of *S. japonicum* (The *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium, 2009). Two years later, a large scale selection had been conducted in order to seek suitable target sequences among those 25 retrotransposons to achieve the sensitive and specific diagnosis of *S. japonica* (Guo et al., 2012). To begin with, primer pairs were designed for the 25 candidates with *Sj*R2, a previously described molecular diagnostic target (Xia et al., 2009; Xu et al., 2010), serving as a positive control. A series of diluted genomic DNA of *S. japonicum* were used as the templates in a PCR assay to amplify

the target fragments and judge the sensitivity of those 25 candidates. The result showed that in addition to *Sj*R2, a new non-LTR retrotransposon named *Sj*CHGCS19, with the PCR product of 303-bp in length, had high sensitivity for detecting *S. japonicum* DNA. Coincidentally, bioinformatics research showed that both *Sj*CHGCS19 and *Sj*R2 have higher genome proportions, repetitive complete copies and partial copies and more active ESTs compared with other candidates in the *S. japonicum* genome, which may also outline the general features of ideal targets for PCR diagnosis of schistosomiasis. Besides, further researches showed that *Sj*CHGCS19 has good specificity except for a cross-reaction with *S. mansoni* and it is also an ideal PCR product for the early diagnosis of schistosomiasis as well as the evaluation of chemotherapy. At last, the clinical utility of *Sj*CHGCS19 was also tested by diagnosing 43 human serum samples from patients of *S. japonica* and 51 serum samples from healthy individuals. As a result, the sensitivity of 97.67% and the specificity of 96.07% fully validated the effectiveness of the *Sj*CHGCS19-based molecular diagnosis of schistosomiasis.

Parasite-derived miRNAs, comparable to genomic DNA, are also present in multiple biofluids of the host, such as plasma, serum, urine, and saliva (Cortez et al., 2011), which implies their potential as biomarkers for the molecular diagnosis of schistosomiasis. Besides, in response to the invasion of pathogens, some characteristic changes of the host's miRNAs profile might appear (Delić et al., 2011), which can also serve as hints of infection event. Nowadays, the available sequences of schistosomal miRNAs, in association with those approaches established for the profiling of miRNAs, e.g., stem-loop RT-PCR, miRNA array, and high-throughput sequencing (Vaz et al., 2010) have paved the way for the future miRNA-based molecular diagnosis of schistosomiasis. For detailed information about the diagnostic and therapeutic value of parasite miRNA, see review (Manzano-Román and Siles-Lucas, 2012).

METABONOMICS-BASED BIOMARKER DISCOVERY

As mentioned above, early schistosomal metabonomics focused on the dynamics of biochemical responses associated with *S. japonicum* or *S. mansoni* infection in multiple biofluids of rodent models. Corresponding progresses made thus far have been reviewed in several papers (Legido-Quigley, 2010; Wang et al., 2010a; Utzinger et al., 2011). To sum up, the metabolic fingerprint of rodents in response to schistosomes infection can be concluded as: reduced levels of the tricarboxylic acid cycle intermediates, stimulated glycolysis, disturbed amino acid metabolism, disturbances in the gut microbiota as well as a phenomenon unique to *S. japonicum* infection, i.e., the inhibition of short-chain fatty acids. Despite the considerable progresses achieved in rodent models, clinical diagnosis of schistosomiasis based on metabonomics still lags behind other -omics methods, because after all, the physiological mechanisms between rodents in the laboratories and humans are far from similar. However, the newly published metabonomic work about human schistosomiasis (Balog et al., 2011) has laid a solid foundation for the clinical application of metabonomic outcomes in the future. Prospective molecular signatures of human *S. mansoni* infection found by this study were primarily related to changes in gut microflora, energy metabolism, and liver function, in line with the earlier findings in experimental animals.

FUTURE DIAGNOSTIC TOOLS OF SCHISTOSOMIASIS AND ADVICE TO BETTER HARNESS -OMICS ACHIEVEMENTS

In our view, future diagnostic tools of schistosomiasis ought to follow three main developmental trends: (1) Field-applicability, test kit protocols should be robust, easy to follow, and produce results within a few minutes without the aid of extra instruments. (2) Non-invasiveness, diagnostic tools should detect specific biomarkers from easy accessible biofluids, e.g., sweat, urea, or saliva. (3) High-throughput, diagnostic kits in the next generation should integrate multiple probes and enable parallel identification of different pathogens.

"-Omics" studies result in a treasure trove of molecular data on genes, proteins, and metabolites. However, identification of effective diagnostic biomarkers from these data pools requires bioinformatics tools based on certain criteria. Further summarization of the general features shared by those currently available diagnostic targets will undoubtedly help us optimize the algorithms for biomarker selection and thereby better utilize the information generated by -omics studies. Besides, some molecular candidates of great diagnostic value are able to be sorted directly by -omics tools based on either their positive interactions with certain biofluids of patients or the changes of their presences or concentrations in response to parasite infection. Schistosomal samples and/or biofluids of patients used in such researches should be chosen and combined according to the prospective utility of a diagnostic tool. Immunomics and metabonomics studies using distinct biological samples should be rigorously designed and carried out in future, in an attempt to seek more diagnostic biomarkers that satisfy variant requirements, e.g., to distinguish infection by different *Schistosoma* species and/or with different worm burden, to discriminate between current infection and previous exposure, etc. Among all available body fluids to date, priority should be given to those that can be sampled without invasive procedures in order to achieve a rapid and safe diagnosis. Last but not least, we should also pay attention to the achievements of those newly established -omics tools, so as to enrich our arsenal of schistosomiasis diagnosis with innovative biomarkers.

Progress in the control and prevention of parasitic diseases is expected to be more rapid and efficient thanks to the achievements of "-omics" researches, benefitting the health of humans and animals alike.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 12 February 2014; accepted: 09 June 2014; published online: 26 June 2014.
*Citation: Wang S and Hu W (2014) Development of "-omics" research in Schistosoma spp. and -omics-based new diagnostic tools for schistosomiasis. Front. Microbiol. **5**:313. doi: 10.3389/fmicb.2014.00313*
This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Microbiology.

frontiers in GENETICS

ORIGINAL RESEARCH ARTICLE
published: 11 June 2014
doi: 10.3389/fgene.2014.00174

Evaluation of the *Schistosoma mansoni* Y-box-binding protein (SMYB1) potential as a vaccine candidate against schistosomiasis

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Schistosomiasis is a neglected tropical disease, and after malaria, is the second most important tropical disease in public health. A vaccine that reduces parasitemia is desirable to achieve mass treatment with a low cost. Although potential antigens have been identified and tested in clinical trials, no effective vaccine against schistosomiasis is available. Y-box-binding proteins (YBPs) regulate gene expression and participate in a variety of cellular processes, including transcriptional and translational regulation, DNA repair, cellular proliferation, drug resistance, and stress responses. The *Schistosoma mansoni* ortholog of the human YB-1, SMYB1, is expressed in all stages of the parasite life cycle. Although SMYB1 binds to DNA or RNA oligonucleotides, immunohistochemistry assays demonstrated that it is primarily localized in the cytoplasm of parasite cells. In addition, SMYB1 interacts with a protein involved in mRNA processing, suggesting that SMYB1 functions in the turnover, transport, and/or stabilization of RNA molecules during post-transcriptional gene regulation. Here we report the potential of SMYB1 as a vaccine candidate. We demonstrate that recombinant SMYB1 stimulates the production of high levels of specific IgG1 antibodies in a mouse model. The observed levels of specific IgG1 and IgG2a antibodies indicate an actual protection against cercariae challenge. Animals immunized with rSMYB1 exhibited a 26% reduction in adult worm burden and a 28% reduction in eggs retained in the liver. Although proteins from the worm tegument are considered optimal targets for vaccine development, this study demonstrates that unexposed cytoplasmic proteins can reduce the load of intestinal worms and the number of eggs retained in the liver.

Keywords: *Schistosoma mansoni*, Y-box-binding protein 1, SMYB1, cytoplasmic antigen, vaccine candidates

INTRODUCTION

Schistosomiasis is the second most important neglected tropical disease causing approximately 280,000 deaths annually (King et al., 2006; Steinmann et al., 2006; Hotez et al., 2008). The disease remains endemic in several developing countries, including Brazil, where *Schistosoma mansoni* is the etiologic agent. The advent of praziquantel was essential to reduce morbidity and mortality due to schistosomiasis. However, the emergence of parasite resistant strains has been reported, raising concerns about the long-term effectiveness of this worldwide available drug (Doenhoff et al., 2002; Hotez et al., 2010). Therefore, the development of new drugs and additional control measures are essential to halt schistosomiasis dissemination. The development of a vaccine that significantly reduces parasitemia is desirable in order to allow a mass treatment with high level of protection and low costs (Chan, 1997; Katz, 1999; McManus, 1999).

Irradiated cercariae used for immunization in experimental animal models regularly induce >80% of protection (Souza et al., 1987; Lin et al., 2011; Tian et al., 2013). However, although some promising antigens have been identified and tested in clinical trials, no effective vaccine against schistosomiasis is currently available. Indeed, most of the studied anti-schistosome targets are tegumental proteins, which directly interact with the host, but consistently do not show satisfactory protection levels (McManus and Loukas, 2008). Consequently, WHO has encouraged tests with new vaccine candidates such as cytoskeletal or cytoplasmic proteins which may be used as part of a multivalent vaccine (Wilson and Coulson, 2006). Additionally, vaccines based on nuclear/cytoplasmic proteins exhibit less chance to trigger an allergic response in the vaccinated individuals (Bethony et al., 2011), as they are not directly exposed to the host immune system.

In this context, the YBPs comprise a family of proteins that are found in most living organisms (Evdokimova et al., 2006) and contain a highly conserved nucleic acid-binding domain, the cold-shock domain (CSD), which possesses great similarity to bacterial cold-shock proteins (Wistow, 1990). In addition to the CSD, proteins from this family have a variable C-terminal TAIL domain predominantly composed of basic or

acid amino acids, which are responsible for either nucleic acid binding or protein-protein interactions (reviewed by Matsumoto and Bay, 2005). YBPs were originally identified as proteins that bind to DNA, RNA, and other proteins (Sommerville and Ladomery, 1996; Matsumoto and Wolffe, 1998; Valadão et al., 2002; Evdokimova et al., 2006; Dong et al., 2009; Mihailovich et al., 2010; Eliseeva et al., 2011). Subsequent studies demonstrated that YB-1, a member of this family, is a major component of ribonucleoprotein particles (mRNPs), working on pre-mRNA splicing, mRNA stability, and translation (Mihailovich et al., 2010; Brandt et al., 2012). Thus, these proteins regulate gene expression and participate in a variety of cellular processes, including transcriptional and translational regulation, induction of DNA repair, cellular proliferation, drug resistance, and stress responses to extracellular signals (Kohno et al., 2003; Mihailovich et al., 2010; Brandt et al., 2012).

In response to stress signals, including low temperatures, drugs that act on DNA, reactive oxygen species, and UV irradiation, the YB-1 protein can translocate from the cytoplasm to the nucleus and participate in gene regulation (Koike et al., 1997; Matsumoto and Wolffe, 1998; Kohno et al., 2003). One of the Y-box protein functions has been elucidated by studies of genes that are repressed in response to YB-1 overexpression in somatic cells. For example, an increase in cellular levels of the human YB-1 protein transcriptionally represses interferon-mediated activation of MHC class II genes (Ting et al., 1994). Subsequent analysis established that YB-1 stimulates the formation of single-stranded regions at the Y-box element (an inverted CCAAT motif) in a MHC class II gene promoter, preventing the loading and/or function of other transacting factors (MacDonald et al., 1995). In addition, it was reported that a synthetic protein can interact with YB-1, stimulating its translocation from the cytoplasm to the nucleus, where YB-1 binds to the promoters of collagen genes and suppresses their transcription, preventing the progression of systemic and hepatic fibrosis (Higashi et al., 2003a,b, 2011; Hasegawa et al., 2009). Currently, a number of genes involved in innate immune response processes and inflammation have been reported to be down- or up-regulated by the YB-1 protein (see the review by Raffetseder et al., 2012).

SMYB1 is a *S. mansoni* protein that belongs to the YBP family and was described by Franco et al. (1997). Due to the similarity between SMYB1 and Y-box proteins from other organisms, and the importance of these proteins in the control of gene expression, our group conducted several studies to characterize the SMYB1 protein. We reported that (i) the protein binds to double- or single-stranded DNA oligonucleotides, with a preference for sequences containing the CCAATT motif, (ii) the protein is expressed in all stages of the parasite life cycle, (iii) SMYB1 interacts with proteins involved in mRNA processing, and (iv) SMYB1 has a cytoplasmic localization (Franco et al., 1997; Valadão et al., 2002; de Oliveira et al., 2004; Rocha et al., 2013). Although the exact function of the SMYB1 protein in this parasite has not been determined, results presented by Valadão et al. (2002) and Rocha et al. (2013) suggested that, while SMYB1 may not act directly as a transcription factor, this protein may be necessary for the regulation of *S. mansoni* gene expression. These studies suggest that SMYB1 can function in the turnover, transport, and stabilization of RNA molecules, acting as RNA chaperones (Valadão et al., 2002; de Oliveira et al., 2004; Rocha et al., 2013). Although intracellular proteins are not usually the first choice of immunogens for vaccination, several extracellular *S. mansoni* proteins have been previously tested with moderate success. We have therefore decided to test SMYB1 as a vaccine candidate against this parasite. To address this matter, we have used Bioinformatics tools to investigate SMYB1 sequence composition and structural features. We have further evaluated the protective efficacy of vaccination with recombinant SMYB1 (rSMYB1) against the *S. mansoni* infection in the murine model.

MATERIALS AND METHODS

ETHICS STATEMENT

Animal experiments were conducted in accordance with Brazilian Federal Law number 11,794, which regulates the scientific use of animals, and United States Institutional Animal Care and Use Committee (IACUC) guidelines. All protocols were approved by the Ethics Committee for Animal Experimentation (CETEA) at Universidade Federal de Minas Gerais under the protocol number 203/2011.

IN SILICO SEQUENCE ANALYSIS

National Center for Biotechnology Information (NCBI) BLAST (Altschul et al., 1990) searches using blastp and PSI-BLAST algorithms were performed against the UniProtKb database (The UniProt Consortium, 2013) using SMYB1 as query to identify possible SMYB1 paralogs with 90% minimal similarity. All subsequent analyses were performed for each of the three identified SMYB isoforms.

Online programs were used to assess functional characteristics of SMYB1. The InterProScan (Zdobnov and Apweiler, 2001) tool was used to recognize different protein signatures (representing protein domains, families, and functional sites) with default parameters. In addition, each SMYB protein isoform was subjected to a conserved domain search (CDS tool) (Marchler-Bauer and Bryant, 2004) from NCBI. Searches were performed against the conserved domain database (CDD v3.10; Marchler-Bauer et al., 2011) with e-values of either 0.01 or 0.001 and with or without applying the low complexity filter. The CDS analysis also points out the known DNA and RNA binding sites present within the predicted domain, by comparing to other proteins that bear the same domain.

The PredictProtein website (Rost et al., 2004) was used to generate information about the protein sequence. Several protein features can be assessed through this webserver, including amino acid composition, predicted protein binding sites and the effect of amino acid substitution. We have submitted all SMYB sequences to the PredictProtein server and retrieved specifically these three results. Protein binding sites are predicted by a machine-learning algorithm indirectly based on 3D structures to identify interacting residues using only the protein sequence as input. The effect of amino acid substitutions for each position is analyzed by exchanging the residue in each position by all other possibilities and investigating the structural/functional effect upon the protein as a whole. The impact of each point mutation is measured by a trained classifier algorithm that takes into account

several features, most importantly from evolutionary information retrieved from sequence alignments. The final output of this method is presented as a heatmap, in which each column represents one position in the protein sequence and each row represents one amino acid. The neutral substitutions are colored from white to dark green, while non-neutral are colored from white to dark red. The original amino acid is marked in black.

Intrinsically disordered regions of the three SMYB isoforms were identified using Disopred (Ward et al., 2004), a trained algorithm that accurately predicts disordered regions by comparison to a dataset of protein regions that could not be solved by X-ray crystallography and, therefore, are largely flexible. False positive rate (FPR) threshold was kept in its default value of 2%.

The secretory or non-secretory nature of the protein was predicted using SignalP 4.1 (Petersen et al., 2011), which identifies signal peptides, and the SecretomeP 2.0 server (Bendtsen et al., 2004), which predicts non-classical protein secretion pathways. Both types of prediction were performed using a default setting score of 0.5. The Euk-mPLoc 2.0 (Chou and Shen, 2010) and TargetP 1.1 Servers (Emanuelsson et al., 2000) were subsequently applied to predict the subcellular locations of SMYB1. GPI-modification sites, mucin type O-glycosylation sites, and N-glycosylation sites were analyzed using the GPI Prediction Server version 3 (Eisenhaber et al., 1999), NetOGlyc 4.0 Server (Steentoft et al., 2013), and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/), respectively. Predicted serine, threonine, and tyrosine phosphorylation sites were obtained using the NetPhos 2.0 Server (Blom et al., 1999).

T and B cell epitopes were predicted based on the amino acid sequences of SMYB1, using prediction tools located at the Immune Epitope Database and Analysis Resource (IEDB-AR), which is a database of experimentally characterized immune epitopes (i.e., B and T cell epitopes) in humans, non-human primates, rodents, and other animal species (http://tools.immuneepitope.org/main/index.html). Linear B cell epitopes were predicted using programs that incorporate solvent-accessible surface area calculations and contact distances into the prediction of B cell epitope potential along the length of the protein sequence. These programs consist of the Emini Surface Accessibility Prediction (Emini et al., 1985), Kolaskar and Tongaonkar Antigenicity (Kolaskar and Tongaonkar, 1990) and the BepiPred 1.0 server (Larsen et al., 2006). To predict T cell epitopes, neural network-based prediction of proteasomal cleavage sites (NetChop) (Nielsen et al., 2005) and T cell epitopes (NetCTL and NetCTLpan) (Larsen et al., 2005; Stranzl et al., 2010) were employed.

CLONING, EXPRESSION, AND PURIFICATION OF RECOMBINANT SMYB1

Initially, the SMYB1 cDNA (Accession no. U39883) was cloned into the pGEM-T Easy vector (Promega). The YB1fwNdeI (5′-CATATGGCGGACACTAGACC-3′) and YB1revHindIII (5′-AAGCTTGATCAGAGAATTTTAAGCGTC-3′) primers were used for SMYB1 amplification from adult worm cDNA, generating an amplification product of 675 bp. The parameters for the PCR reaction were as follows: 1 cycle at 95°C for 6 min followed by 25 cycles of 1 min at 95°C, 1 min at 58°C, 1 min at 72°C and a final cycle of 5 min at 72°C. The recombinant pGEM-SMYB1 vector was then digested with the enzymes *Nde*I and *Hin*dIII and the recovered insert was subcloned into the pET28aTEV vector, in-frame with the six histidine N-terminal (6xHis) tag. DNA sequencing was performed to confirm the presence and the correct orientation of the SMYB1 cDNA. *Escherichia coli* BL21 was transformed with the recombinant plasmid (pET28a-SMYB1) and grown in Circlegrow medium (MP Biomedicals) supplemented with kanamycin (100 μg/ml), at 37°C, 180 rpm. Bacterial growth was monitored at OD600 nm until reach 0.4–0.6 and the expression of rSMYB1 was induced by the addition of 0.5 mM IPTG. After 4 h of induction, the bacterial cells were harvested by centrifugation at 7690 g for 20 min. The pellet was resuspended in 50 mL of column buffer (20 mM sodium phosphate; 300 mM NaCl; 20 mM imidazole, pH 7.4; 10% glycerol). Lysozyme (100 μg/mL) was subsequently added, and the cells were incubated for 15 min. The cells were then subjected to 3 cycles of heat shock (−80°C/37°C), followed by three 15 s cycles of sonication (Fisher Scientific) and three rounds of centrifugation at 5940 g for 20 min. The protein was purified from the supernatant by affinity chromatography on a HisTrap HP 5 mL Ni-Sepharose column (GE Healthcare) under denaturing conditions using the ÄKTA Prime Plus Liquid Chromatography System (GE Healthcare), according to the manufacturer's instructions. Fractions containing rSMYB1 were dialyzed against Tris-NaCl buffer (50 mM Tris; 20 mM NaCl, pH 7.4), which was changed every 12 h. The dialysis was performed for 36 h at 4°C using a >12 kDa dialysis tubing cellulose membrane (Sigma Aldrich). The protein was aliquoted and stored at −80°C until use. Protein concentration was determined using Bradford's method (Bradford, 1976). The recombinant protein was used as an antigen for immunization and in immunological experiments.

SDS-PAGE AND IMMUNOBLOTTING

SDS-PAGE of purified rSMYB1 was performed using 12% gels, and the gels were electroblotted onto nitrocellulose membranes for 30 min at 20 V using a semi-dry system (Bio-Rad). The membranes were blocked with phosphate-buffered saline (PBS) (130 mM NaCl, 2 mM KCl, 8 mM Na2HPO4, 1 mM KH2PO4) plus 0.05% Tween 20 (PBS-T) containing 5% dry milk (p/v) for 16 h at room temperature. The membrane was subsequently incubated in 1:2000 dilutions of an anti-His antibody (GE Healthcare) and peroxidase-conjugated anti-mouse IgG (Sigma Aldrich) in PBS-T for 1 h at room temperature. After washes using PBS-T, the membrane was developed using 3,3′-diaminobenzidine (Sigma Aldrich), according to the manufacturer's protocol. After developing, the membrane was washed using distilled water and dried on filter paper.

IMMUNIZATION OF MICE AND MEASUREMENT OF SPECIFIC ANTI-rSMYB1 ANTIBODIES

Female C57BL/6 mice ($n = 10$, per group) between 6 and 8 weeks of age were obtained from the Universidade Federal de Minas Gerais (UFMG) animal facility and supplied with commercial food and water *ad libitum*. Mice were subcutaneously injected in the nape of the neck with 25 μg of rSMYB1 on days

0, 15, and 30. The vaccine was formulated with the recombinant protein emulsified in complete Freund's adjuvant (CFA) (Sigma Aldrich) for the first immunization and incomplete Freund's adjuvant (IFA) (Sigma Aldrich) for subsequent immunizations. In the control group, Tris-NaCl buffer with Freund's adjuvant was administered using the same immunization protocol.

On the tenth day after each immunization, blood was collected from each experimental group by retro-orbital bleeding. The levels of specific anti-rSMYB1 antibodies were measured by indirect ELISA. Briefly, Maxisorp 96-well microtiter plates (Nunc) were coated with 5 μg/mL rSMYB1 in carbonate-bicarbonate buffer, pH 9.6, for 16 h at 4°C. The plates were then blocked for 2 h at room temperature with 200 μl of PBS-T plus 10% fetal bovine serum (FBS) (Life Technologies) per well. The serum from each mouse was diluted 1:100 in PBS-T, and a 100-μl sample was added to each well and incubated for 1 h at room temperature. Plate-bound antibody was detected using peroxidase-conjugated anti-mouse IgG, IgG1, and IgG2a (Sigma Aldrich) diluted to concentrations of 1:5000, 1:10000, and 1:2000 in PBS-T, respectively. Color reactions were developed by the addition of 100 μL per well of 200 pmol o-phenylenediamine (OPD) (Sigma Aldrich) in citrate buffer, pH 5.0, plus 0.04% H_2O_2 for 10 min. The reactions were stopped with 50 μL of 5% sulfuric acid per well. The plates were read at 492 nm using an ELISA plate reader (Bio-Rad).

CHALLENGE INFECTION WITH *S. MANSONI* AND WORM BURDEN RECOVERY

Cercariae of *S. mansoni* (LE strain) were maintained routinely in *Biomphalaria glabrata* snails at the Centro de Pesquisas René Rachou - Fiocruz (CPqRR) and prepared by exposing infected snails to light for 2 h to induce shedding. Cercariae numbers and viability were determined using a light microscope prior to infection. Challenge infection was performed 10 days after the final immunization. Mice were anaesthetized with 90 mg/kg of ketamine and 10 mg/kg of xylazine. The mice abdomens were shaved and they were exposed percutaneously to 100 cercariae of *S. mansoni* in water for 1 h using the ring method (Smithers and Terry, 1965). Forty-five days after challenge (DAC), the mice were sacrificed and the adult worms were perfused from the portal veins (Fonseca et al., 2004). Two independent experiments were performed to determine protection levels and 10 mice per group were used.

Protection was calculated by comparing the number of worms recovered from each vaccinated group with its respective control group, using the following formula: $PL = (WRCG - WREG) \times 100/WRCG$, where PL, protection level; WRCG, worms recovered from control group; and WREG, worms recovered from experimental group.

QUANTIFICATION OF *S. MANSONI* EGGS RETAINED IN THE LIVER

Quantification of *S. mansoni* eggs retained in the liver was performed according to the protocol described by Cheever (1968). To count the number of eggs in the liver, the organ was recovered from each experimental mouse, weighted and placed into 20 mL of a 5% KOH solution (p/v) in a 50 mL tube. Digestion occurred at room temperature for 48 h, and the samples were subsequently mixed thoroughly. The solutions were centrifuged for 3 min at 200 g, and the pellets were resuspended in 20 mL PBS and vortexed. This step was repeated three times. After the last wash, eggs were resuspended in 5 mL of 10% buffered formaldehyde in PBS and maintained at room temperature until counting. An average of three counts was obtained per 50 μL solution to estimate the number of eggs per gram of tissue. Protection was calculated by comparing the number of eggs recovered from the vaccinated group to the number of eggs recovered from its respective control group, using the same formula used for adult worms.

HEPATIC GRANULOMA ANALYSIS

Liver sections from mice of control and vaccinated groups and infected with 100 cercariae were collected 45 days post-infection to evaluate the effect of immunization in granuloma formation. The liver sections removed from the central part of the left lateral lobe were fixed with 10% buffered formaldehyde in PBS. Histological sections were performed using microtome (4 μm) and stained in a slide with Gomory's trichromic. The granulomas were counted in Axiolab Carl Zeiss microscope using 10× objective lens. All slides were digitized by the Canon Lide 110 scanner, in 300 dpi resolution. The pixels of each histological section were fully screened, with subsequent creation of a binary image and the total area of the cut was calculated. The area of the lower cutoff was used as a minimum standard of tissue to be statistically analyzed. The results were expressed by the number of granulomas per area of liver (mm^2). The area of granulomas was obtained through the KS300 software contained in Carl Zeiss image analyzer. Fifteen granulomas from each mouse with a single well-defined egg were randomly chosen at a microscope with 20× objective lens and scanned through a Q-Color3 microcamera (Olympus). Using a digital pad, the total area of granulomas was measured and the results were expressed in square micrometers (μm^2).

HUMORAL RESPONSE AGAINST rSMYB1 AND *S. MANSONI* ANTIGENS AFTER CHALLENGE

Following immunization, blood was collected using the previously described protocol (see section Immunization of Mice and Measurement of Specific anti-rSMYB1 Antibodies) at day 0 (i.e., challenge) and day 45 of infection (i.e., sacrifice). Measurements of specific anti-SMYB1, anti-*Schistosoma* worm antigen protein (SWAP), and anti-soluble egg antigen (SEA) IgG, IgG1, and IgG2a antibodies in the sera were performed using indirect ELISA, as previously described.

STATISTICAL ANALYSIS

Statistical analysis was performed using Student's *t*-test in the GraphPad Prism 5.0 software package (La Jolla, CA, USA).

RESULTS

IN SILICO ANALYSES OF SMYB1 SEQUENCE

In *S. mansoni*, the SMYB1 protein (predicted molecular weight: 23805.20 Da, theoretical pI: 10.21) is encoded by the Smp_097800 gene, which produces three transcript isoforms: Smp_097800.1 (SMYB1), Smp_097800.2 (SMYB2), and

Smp_097800.3 (SMYB3) derived from alternative splicing (**Figure 1A**). BLAST searches using blastp and PSI-BLAST algorithms against the UniProtKb database revealed a paralog protein in *S. mansoni* (SMYBX_putative), encoded by the Smp_097750 gene, which produces a single transcript isoform (Smp_097750.1) (**Figure 1B**). Global alignment shows that the SMYB proteins are much conserved (more than 90% identity). The N-terminal region (CSD) is more conserved among all sequences, consistent with the fact that all Smp_097800 derived isoforms share the first 156 amino acids, and only diverge in their C-terminal domain. Interestingly, the Smp_097750 derived isoform has an almost perfectly conserved CSD region (**Figure 1C**).

The InterProScan tool identified an N-terminal nucleic acid-binding OB-fold domain (IPR012340) in the SMYB isoforms (**Figure 1C** and Figure S1), which is found in the Y-box binding protein subfamily (PTHR11544:SF6). The presence of this domain was also confirmed by the CDS tool with high confidence (*e*-vaule of 0.001). The CDS tool has also identified a C-terminal

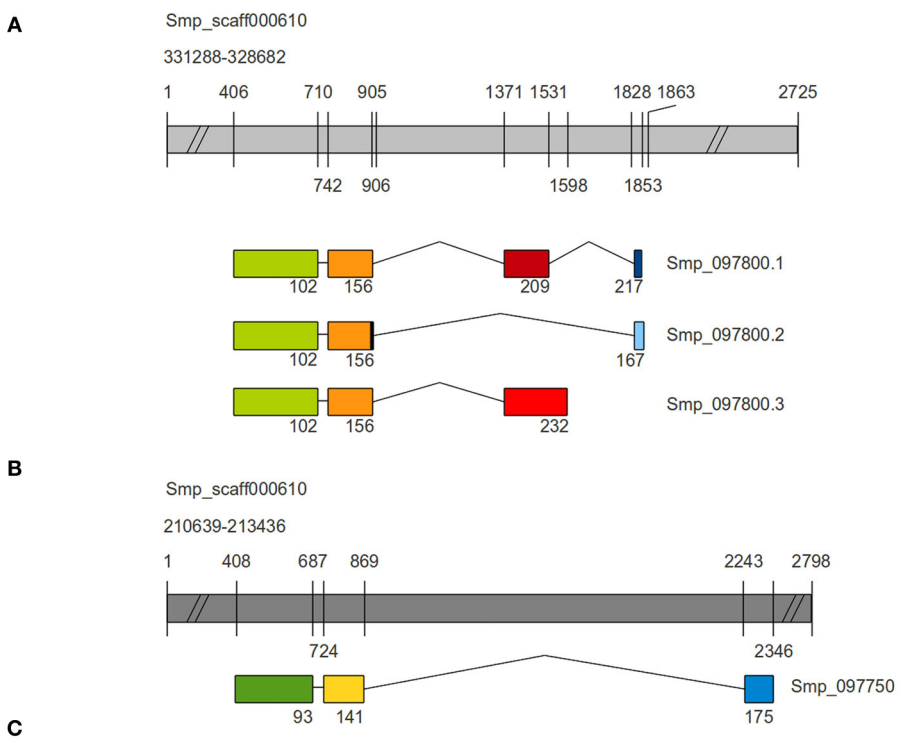

FIGURE 1 | Comparison between SMYB1 and other SMYB isoforms. The coding sequences for all SMYB proteins were aligned to two segments of the *S. mansoni* genome scaffold 000610, in order to identify exonic and intronic portions of the genes. Genes are represented by gray bars, while exons are represented by colored boxes and introns by connecting lines. Numbers depicted on the gray bars are relative to nucleotide positions and numbers below the exons are relative to amino acid positions on the resulting protein sequence. **(A)** For SMYBX (Smp_097750), there are three exons and the third (C-terminal, blue box) one is separated from the first two (dark green and yellow boxes) by a very long intron. **(B)** Regarding its paralogs SMYB1 (Smp_097800.1) is formed by four exons and the first two (green and orange boxes) are shared with isoforms SMYB2 (Smp_097800.2, with one extra nucleotide at the end of the second exon) and SMYB3 (Smp_097800.3). While the SMYB2 isoform contains an exon (light blue box) similar to SMYB1 exon 4 (navy blue box), but longer (and in a different reading frame), SMYB3 contains a third exon (light red) which is similar to SMYB1 exon 3 (dark red), but longer. **(C)** Muscle alignment of SMYB1 (Q27277), SMYB2 (G4LXD2), SMYB3 (G4LXD0), and the SMYBX_putative (G4LXC4). Disordered regions are highlighted in red, according to Disopred predictions and the conserved domain is underlined in blue.

API5 domain (apoptosis inhibitor domain 5) approximately localized between residues 140 and 200 on the longer isoforms (SMYB1 and SMYB3) although with low confidence (*e*-value of 0.01). Further analysis may confirm this as an actual conserved domain or just an artifact (Figure S1).

The prediction of intrinsically disordered regions has characterized SMYB isoforms as mostly disordered proteins. It is interesting to observe that the conserved CSD is located away from the disordered regions (Figure S1). An additional region where the disorder probability suddenly drops (flanking the residue 180) is an interesting feature to be further investigated (Figure S1). Another interesting finding regarding the disorder is its relation to protein-binding residues. For all isoforms, predicted protein binding sites range from residues 1 to ∼25, ∼110 to the end of the sequence and position 65, which is the only predicted binding site out of the disordered region (Figure S1 and Supplementary Material).

When observing the SNAP results presented in Figure S1, one can easily identify the first ∼20 N-terminal residues as predicted to contribute very little to the structure and function of SMYB isoforms, since all simulated mutation in such positions seem to have no effect to the proteins. On the other hand, the region where the CDS domain is located is the most important and mutations in this region can easily have a negative effect to protein structure and function. This is expected, since this is the only structured region of the proteins. Accordingly, the nucleic acid binding site regions are the most conserved within this domain, since the heatmap is dark red around these sites.

SMYB1 was predicted to be located in the cytoplasm and nucleus of *S. mansoni* cells, using the Euk-mPLoc program. No cleavage sites or N-terminal presequences consistent with a mitochondrial targeting peptide or secretory pathway signal peptide were identified using the TargetP Server. In addition, the SecretomeP server revealed SecP scores below the cutoff score (0.50), indicating a low possibility of secretion by the non-classical pathway.

Additional Bioinformatics analyses of domain prediction, protein disorder, protein structure, and molecular interactions, as well as putative post-translational modifications (GPI modification, glycosylation, and phosphorylation sites) and B-cell and T-cell type epitope predictions for SMYB1 are presented and briefly discussed in the Supplementary Material (Table S1 and Figures S1, S2).

EXPRESSION AND PURIFICATION OF RECOMBINANT SMYB1

The SMYB1 gene was cloned into the pET28a expression vector, and the recombinant protein was successfully expressed as a 6xHis tag fusion protein. The transformed bacterial cells were treated with lysozyme, submitted to heat shock and sonication treatments, and the lysates were separated into soluble and insoluble fractions (**Figure 2A**). The protein was purified from the soluble fraction by affinity chromatography using His-binding columns under denaturing conditions (**Figure 2B**). The protein was then refolded by dialysis against Tris-NaCl buffer, with an approximate yield of 11 mg of protein/liter. The purity of the recombinant SMYB1-6xHis tag fusion protein was assessed using SDS-PAGE and Western blotting analysis with an anti-His antibody (**Figure 2C**), which revealed a protein of approximately 30 kDa.

HUMORAL RESPONSES TO rSMYB1

C57Bl/6 mice were immunized with three doses of rSMYB1 formulated with Freund's adjuvant, and the level of specific anti-rSMYB1 antibodies in the sera from the immune and placebo groups was evaluated using ELISA (**Figure 3**). Significant levels ($p < 0.01$) of specific anti-rSMYB1 IgG antibodies were detected after the first immunization, and these antibodies remained at a high levels after the second and third immunizations. To

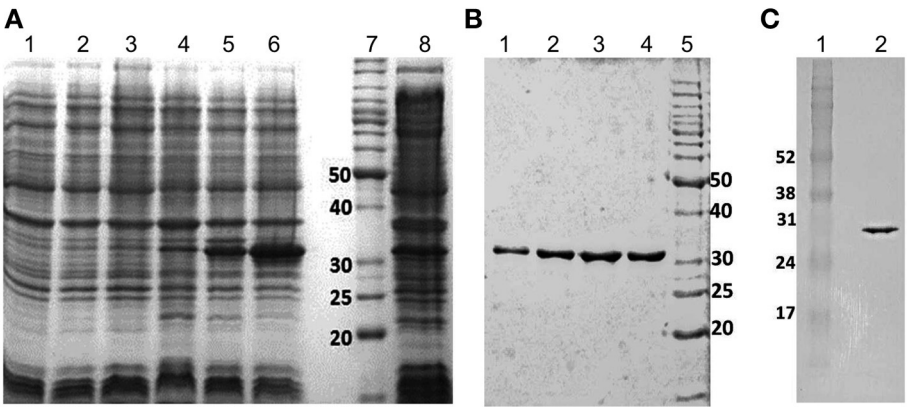

FIGURE 2 | Expression and purification of rSMYB1 as a 6xHis fusion protein. (A) Coomasie blue-stained 12% SDS-PAGE profile of *E. coli* BL21 expressing the pET28a-SMYB1 construct. Lanes: (1) lysate of induced culture—0 h; (2) lysate of induced culture—1 h; (3) lysate of induced culture—2 h; (4) lysate of induced culture—3 h; (5) lysate of induced culture—4 h; (6) soluble fraction (SF) of induced culture lysate; (7) molecular weight marker (BenchMark Protein Ladder, Invitrogen); (8) non-soluble fraction of induced culture lysate (pellet). **(B)** Coomasie blue-stained 12% SDS-PAGE profile of the fractions of purified recombinant SMYB1. Lanes (1–4) SMYB1 fractions; lane (5) molecular weight marker (BenchMark Protein Ladder, Invitrogen). **(C)** Western blotting analysis of purified recombinant SMYB1 using an anti-His antibody (GE Healthcare). Lane (1) molecular weight marker (Amersham Full Range Rainbow Molecular Weight); lane (2) purified rSMYB1.

determine the isotype of the antibody produced after immunization, IgG1 and IgG2a antibodies specific to rSMYB1 were also analyzed. The results revealed that rSMYB1 stimulates an IgG1 antibody response ($p < 0.05$) after the second dose (**Figure 3**). In the placebo group, no significant differences in specific IgG, IgG1, or IgG2a antibody levels were observed after immunization (data not shown).

S. MANSONI ADULT WORM RECOVERY

To determine the protective potential of rSMYB1, immunized mice were challenged with 100 *S. mansoni* cercariae. The worms were recovered by perfusion 6 weeks after challenge, and the results were expressed as the mean worm burden (mean ± *SD*) as presented in **Table 1**. The animals immunized with rSMYB1 in Freund's adjuvant exhibited a 26% reduction in adult worm burden recovered from the mesenteric veins when compared to the control group ($p > 0.05$). No differences in male/female proportion were observed between the placebo and immune groups (data not shown). Similar results were observed in two independent experiments.

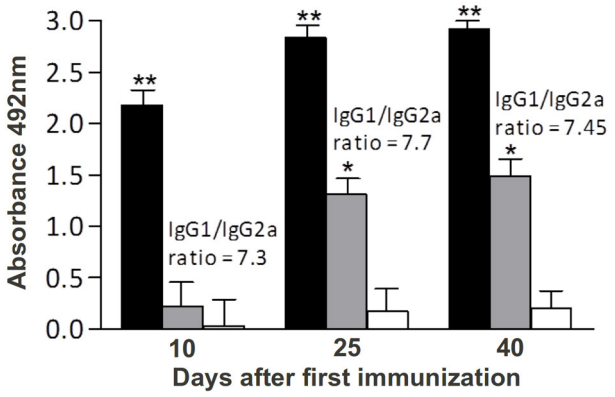

FIGURE 3 | Kinetics of specific anti-rSMYB1 IgG, IgG1, and IgG2a antibodies in sera from mice vaccinated with rSMYB1. Sera were collected from 10 immunized mice per group prior to the first immunization and at days 10, 25, and 40 after the first immunization and assayed by ELISA. The results are presented as the mean absorbance at 492 nm for each isotype on each day of sera collection. Asterisks indicate statistically significant differences between the vaccinated and preimmune groups: $*p < 0.05$; $**p < 0.01$. Black bars: IgG antibody. Gray bars: IgG1 antibody. White bars: IgG2a antibody. The results are representative of two independent biological replicates.

Table 1 | Worm burden and protection level in mice vaccinated with the rSMYB1 protein.

Group	Worm burden (mean ± *SD*)	Protection
Tris-NaCl + CFA/IFA (placebo)	51.50 ± 26.64	–
rSMYB1 + CFA/IFA (immune)	38.11 ± 10.78	26%

CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant. No statistically significant differences were observed between groups ($p > 0.05$). The data are representative of two independent biological assays.

QUANTIFICATION OF *S. MANSONI* EGGS RETAINED IN THE LIVER

In addition to worm counting, we evaluated the number of *S. mansoni* eggs retained in each gram of liver. The immunized group retained 28% less eggs in the liver than the placebo group ($p > 0.05$) (**Figure 4**). We have also measured the number of eggs layed by female adult worm recovered before and after immunization and found a 5.5% decrease in the number of eggs per female on the immunized group (the average was of 769.20 eggs/female on the placebo group against 726.69 eggs/female on the immunized group). Therefore, these result points to a combination between diminished egg production per female and decreased number of adult parasites in the host after immunization.

HISTOPATHOLOGICAL ANALYSIS

Histopathological analysis showed significantly fewer granulomas in the liver of animals immunized with rSMYB1 ($p < 0.05$) (**Figure 5A**). An associated decrease in the area of granulomas in the immunized mice group compared to the placebo group ($p < 0.05$) was also observed (**Figures 5B**, **6**). However, no significant decrease in the area of fibrosis was detected when the two groups were compared ($p > 0.05$) (**Figure 5C**).

HUMORAL RESPONSE AGAINST rSMYB1 AND *S. MANSONI* ANTIGENS AFTER CHALLENGE

Levels of specific antibodies produced in response to the purified rSMYB1 protein in each group of mice after challenge were determined using ELISA. Surprisingly, the levels of rSMYB1-specific IgG, IgG1, and IgG2a antibodies in the immune group decreased after the third dose of the vaccine ($p > 0.05$) (**Figure 7**). In contrast, the placebo group exhibited increased levels of all antibodies against the protein. No statistically significant differences were observed between the immune and placebo groups at 45

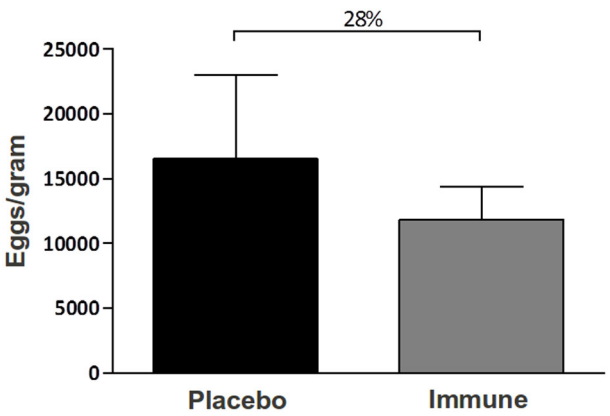

FIGURE 4 | Counting of the number of *S. mansoni* eggs retained in the liver of immunized mice. The number of *S. mansoni* eggs in 10 mice immunized with Tris-NaCl + adjuvant (placebo group) or with rSMYB1 + adjuvant (immune group) were counted. The infection developed for 45 days prior to sacrifice. The liver was digested for 48 h in a 5% KOH solution (p/v), and the eggs were maintained in 10% buffered formaldehyde until counting. The results are presented as the mean number of eggs per gram of liver. No statistically significant differences were observed between groups ($p > 0.05$). The results are representative of two independent biological experiments.

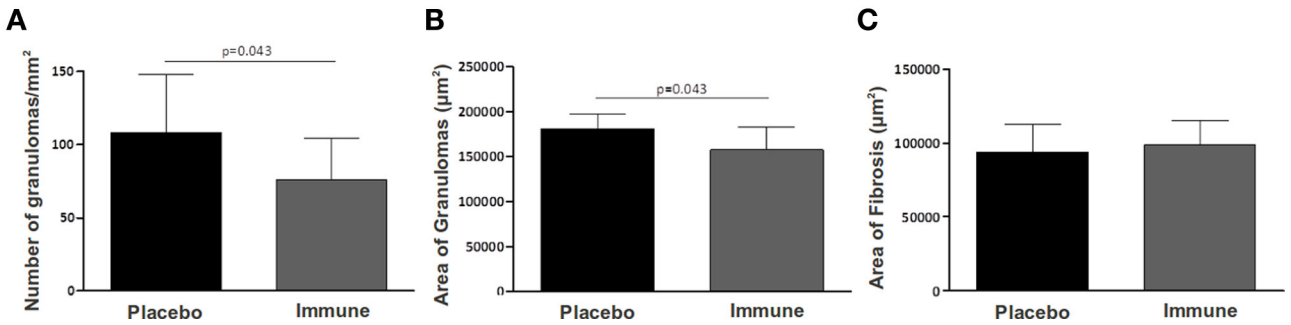

FIGURE 5 | Liver pathology measured in rSMYB1 vaccinated mice. (A) Number of granuloma per area of liver (mm^2), **(B)** area of granuloma (μm^2), and **(C)** area of fibrosis (μm^2) were measured in rSMYB1 immunized mice and control group. Data are expressed as means ± SD.

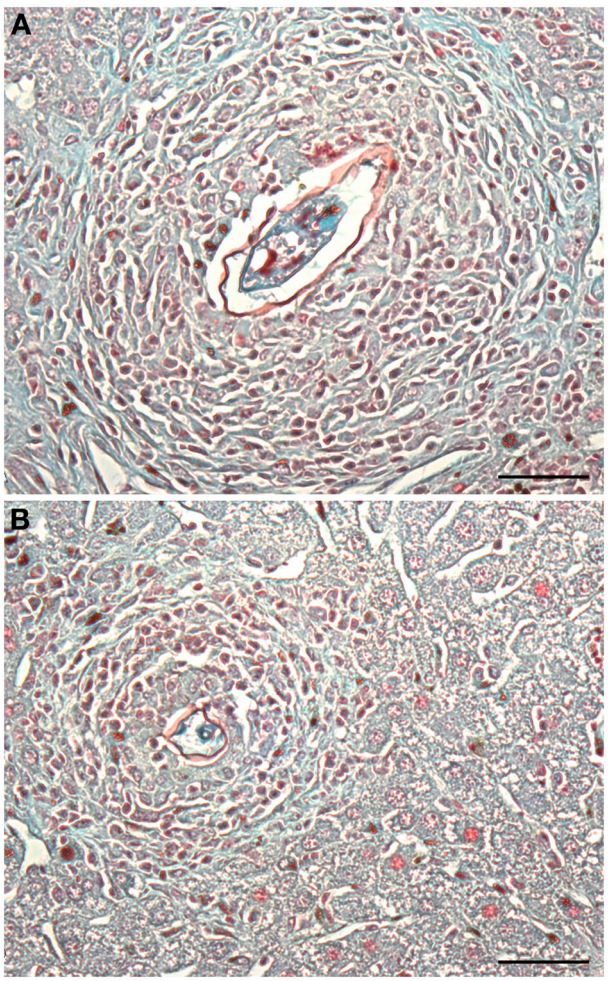

FIGURE 6 | Histological analysis of liver tissue from mice immunized with rSMYB1. Animals were sacrificed 45 days post-infection and their livers were washed with PBS and stored in formaldehyde until histological procedures. Fixed livers were sliced using microtome (4 µm) and stained with Gomory's trichromic. **(A)** Liver from the control group and **(B)** liver from rSMYB1 immunized mice. Scale bar: 50 µm. The granulomas were counted in Axiolab Carl Zeiss microscopy using 10× objective lens.

DAC ($p > 0.05$). The observed anti-rSMYB1 IgG1/IgG2a ratio decreased in the immunized mice.

To measure IgG, IgG1, and IgG2a antibodies against the specific *S. mansoni* antigens SWAP and SEA, the sera from vaccinated mice in each group were tested using ELISA (**Figure 7**). No specific anti-SWAP and anti-SEA IgG, IgG1, and IgG2a antibodies were detected before or during the immunizations (data not shown). After challenge, both groups developed significant levels of specific anti-SWAP and anti-SEA (**Figure 7**) IgG antibodies ($p < 0.01$). With respect to isotype, both groups developed a predominantly IgG1 antibody response ($p < 0.05$) against the SWAP antigen but not against the SEA antigen. No differences in IgG2a antibody response against these antigens were observed between the immune and placebo groups.

DISCUSSION

The long-term effective control of schistosomiasis will only occur as a result of combined vaccination and chemotherapy strategies with sanitation and public health control measures. Although evidences indicate that chemotherapy using praziquantel is effective in reducing the intensity of infection, as reinfection has been observed after chemotherapy, the use of this control strategy alone has been questioned (Wilson and Coulson, 2006). The irradiated cercarial vaccine elicits >80% protection in rodents and primates and other antigens identified in analyses of the *Schistosoma* proteome, transcriptome, glycome, and immunome, also exhibit protective potential (Oliveira et al., 2008). Nevertheless, the effectiveness of recombinant vaccines rarely exceeds 40% (McManus and Loukas, 2008; McWilliam et al., 2012), although new findings from El Ridi and collaborators (El Ridi and Tallima, 2013; El Ridi et al., 2014) are promising and depict a decrease of ∼70–80% in worm burden using papain as adjuvant and focusing on *S. mansoni* cysteine peptidases as antigens.. To date, vaccine candidates have been assessed using omics-derived high throughput approaches, such as proteomics, immunomics, and vaccinomics with promising results (DeMarco and Verjovski-Almeida, 2009; Loukas et al., 2011; McWilliam et al., 2012).

Many studies focus on tegument proteins as potential drug/vaccine targets because the tegument is a dynamic layer that represents the primary host-parasite interface and has close

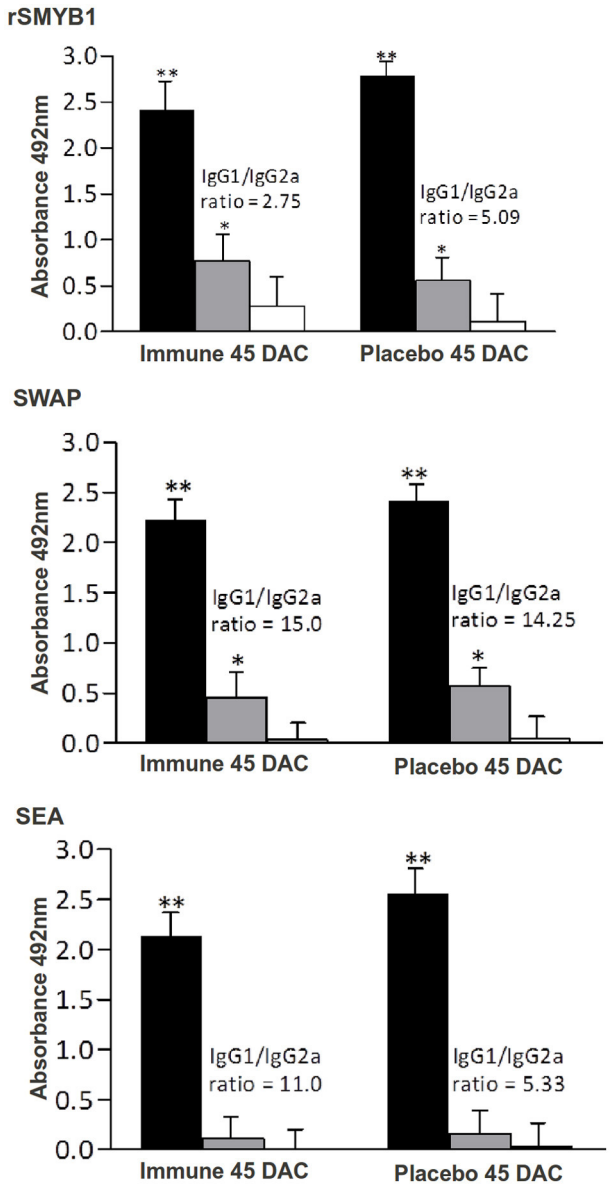

FIGURE 7 | Kinetics of specific anti-rSMYB1, anti-SWAP and anti-SEA IgG, IgG1, and IgG2a antibodies. The presence of antibodies was measured in sera from 10 mice immunized with Tris-NaCl + adjuvant (placebo group) or with rSMYB1 + adjuvant (immune group). Sera were collected prior to sacrifice 45 days after challenge and assayed by ELISA. The results are presented as the mean absorbance at 492 nm for each immunoglobulin isotype. Asterisks indicate statistically significant differences between the vaccinated and pre-immune groups: $*p < 0.05$, $**p < 0.01$. DAC, days after challenge. Black bars: IgG antibody. Gray bars: IgG1 antibody. White bars: IgG2a antibody. The results are representative of two independent biological experiments.

proximity to the host blood and immune system (Jones et al., 2004; Pearce and Freitas, 2008; DeMarco and Verjovski-Almeida, 2009; Han et al., 2009; Loukas et al., 2011). Other studies focus on excretory/secretory (ES) proteins, molecules known to be released from live worms in the tissue culture and that may be secreted into host tissues as the parasites move along the host body, feed, and produce eggs (Loukas et al., 2011). According to McManus and Loukas (2008), the apical membrane proteins expressed on the surfaces of the schistosomulum and the adult worm are the preferred vaccine targets. Therefore, the use of extracellular antigens for vaccine production is accompanied by inherent problems, for instance, the difficulty to produce recombinant proteins, since the majority of these antigens is processed through the classical secretory pathway and is subject of complex post-translational modifications, (e.g., glycosylation, specific processing, and disulfide bonds formation). Additionally, most antigens tested in WHO trials and by other groups are cytosolic or cytoskeletal components (e.g., paramyosin, Sm14, and GST) (Wilson and Coulson, 2006; McManus and Loukas, 2008; Oliveira et al., 2008). To our knowledge no study exploring the potential of a nucleic acid-binding protein as a *S. mansoni* vaccine candidate has been published. This is the first attempt to characterize such a protein as an antigen and to evaluate its protective efficacy as a vaccine against *S. mansoni* infection in the murine model.

In 2005, Carl and collaborators have stated that most nuclear systemic autoantigens contain long regions of structural disorder. They have studied properties of intrinsically disordered proteins in order to make connections linking disorder to antigenicity. The authors state that the amino acid composition of disordered regions (usually rich in Arg, Gly, Ser, Pro, Glu, Lys, Gln, and Ala residues) leads to a highly charged and low complexity molecule, typical properties of autoantigens (Plotz, 2003). Another property of autoantigens is their capacity to bind nucleic acids, as described by Plotz and cited by Carl and collaborators. Additionally, Plotz listed phosphorylation as a strong feature of autoantigens. All of these factors, namely enrichment in six of the listed amino acids (Arg, Gly, Ser, Pro, Glu, Lys), low complexity regions (such as repetitive sequence patterns), nucleic acid binding capacity and the abundance of phosphorylation sites (10 predicted) can be observed in the SMYB1 protein, thus corroborating its putative antigenic potential.

Although the Euk-mPLoc program predicted the SMYB1 localization in the cytoplasm and nucleus of cells, our group has previously demonstrated that this protein is predominantly located in the cytoplasm of cells from different life cycle stages of *S. mansoni*, suggesting that SMYB1 is probably acting in RNA metabolism in the cytoplasm. We also showed the presence of SMYB1 near the tegument in adult worms proposing an action on the translational regulation of tegument proteins (Rocha et al., 2013). Intrinsically disordered proteins have recently been characterized as the prevalent type of RNA and protein chaperones (Tompa and Csermely, 2004). Accordingly, it has been shown that YBPs and other cold-shock proteins typically act as chaperones that maintain mRNA in a single-stranded conformation to sustain the expression of genes that are necessary for cell growth, proliferation, and transformation (Jiang et al., 1997; Matsumoto and Wolffe, 1998; Salvetti et al., 1998; Tanaka et al., 2004; Evdokimova et al., 2006). YBPs are thought to play roles in a wide variety of responses to environmental stresses (Kohno et al., 2003). As such, SMYB1 localization in the cytoplasm of tegumental cells reinforces its

importance as a protein that acts responding to the stressing host environment.

Molecules that contain signal peptides or signal anchors are predicted to be excreted, secreted or membrane-anchored, directly interacting with the host immune system and, as stated above, constitute relevant targets for schistosome vaccines. The combined Bioinformatics results obtained in this study suggest that the SMYB1 protein is not secreted. However, Frye et al. (2009) reported that human YB-1 is secreted from cells during inflammatory stress after treatment with lipopolysaccharide, hydrogen peroxide or TGFβ. In these cases, YB-1 is secreted not via the classical mechanism of protein secretion (i.e., via the Golgi apparatus and endoplasmic reticulum) but by a non-classical mechanism inside endolysosomal vesicles (Frye et al., 2009; Eliseeva et al., 2011). The question of whether SMYB1 is secreted or not needs further experimental investigation.

We reported here the successful cloning of SMYB1 cDNA into the pET28a vector and the expression of rSMYB1 in the soluble fraction of bacterial lysates. The discrepancy between the ∼30 kDa protein molecular mass value calculated from SDS-PAGE and the ∼24 kDa protein molecular mass value predicted from the cDNA is typical of Y-box proteins and related to the anomalous electrophoretic properties of these proteins (Deschamps et al., 1992) or to post-translational modification, such as phosphorylation (Salvetti et al., 1998). We subsequently evaluated the antigenicity of the protein by investigating the murine humoral immune response to rSMYB1 and the impact of its immunization on adult worm and egg burden in mice challenged with 100 cercariae of *S. mansoni*. Recent data suggested that the establishment of a robust humoral response is likely the key for generating maximal immunity against schistosomes (Wynn and Hoffmann, 2000). A primary obstacle to the development of a schistosome vaccine is the lack of available knowledge concerning the type of immune response that should be induced. In the irradiated cercariae vaccination model, above 80% protection can be granted by a Th1, a Th2, or a mixed Th1/Th2 immune response (Wynn and Hoffmann, 2000). However, with respect to recombinant proteins, Th1-inducing antigens have been reported to confer protection against *Schistosoma* infection in the mouse model (Jankovic et al., 1996; Mountford et al., 1996; Zhang et al., 2001; Fonseca et al., 2004; Varaldo et al., 2004; Li et al., 2005; Cardoso et al., 2008; Garcia et al., 2008).

In this study, C57Bl/6 mice immunized with rSMYB1 exhibited high levels of specific anti-SMYB1 IgG antibodies that emerged after the first immunization. Specific anti-SMYB1 IgG1 antibodies predominated over IgG2a antibodies, particularly after the second immunization. However, the IgG1/IgG2a ratio was reduced after the last immunization (i.e., during the challenge period). Antibody levels correlated with protective efficacy in our study. The antibody levels developed by mice immunized with rSMYB1 reduced in 26% the number of adult worm burden and in 28% the eggs/granuloma trapped in the liver. A critical issue in vaccine design is the use of an appropriate adjuvant to induce the suitable immune response. Although the CFA adjuvant, which triggers a Th1 response, cannot be used in humans (Heegaard et al., 2011), it is widely utilized in initial immunization trials. Further experiments combining rSMYB1 with suitable adjuvant formulations for use in humans should be performed. In this sense, an interesting strategy would be to use papain as adjuvant, given that recently published articles have described very high protection rates related to the use of such molecule in vaccine candidates (El Ridi and Tallima, 2013; El Ridi et al., 2014).

S. mansoni adult worms live in the blood essentially unrecognized for many years, whereas schistosome eggs are a prominent target of the host immune response. In the first weeks of murine *S. mansoni* infection, a Th1 immune response is observed and the eggs deposited in the blood vessels by females that pass to the endothelial barrier and become trapped in the liver are immediately targeted by recruited immune cells that consist primarily of T-cells, eosinophils, and macrophages (Pearce and MacDonald, 2002; Wynn et al., 2004). Histopathology results show that in the initial phase of infection vaccination with SMYB1 seems to interfere with cell recruitment and migration in the liver. Consequently, the resulting granulomas, although presenting the same area of fibrosis, were fewer when compared to unvaccinated animals, showing the protective potential of the protein in the initial liver pathology.

Although tegument proteins are considered the main targets for vaccine development (Bergquist et al., 2002; McManus and Loukas, 2008), this study demonstrates that a cytoplasmic protein has the potential to be used as an immunogen, as we showed that SMYB1 could reduce the load of intestinal worms and eggs retained in the liver when it was used in vaccination trials and also that the protection levels achieved by SMYB1 are comparable to those obtained with other tegument and cytoskeleton proteins.

AUTHOR'S CONTRIBUTIONS

Sílvia R. C. Dias, Mariana Boroni, Thomaz L. Dias, Daniela de Laet Souza, Fabrício M. S. Oliveira, Elizângela A. Rocha, Mainá Bitar, Andrea M. Macedo, Carlos R. Machado, Marcelo V. Caliari, and Glória R. Franco contributed to the conception and design of the experiments; Sílvia R. C. Dias, Mariana Boroni, Thomaz L. Dias, Daniela de Laet Souza, Fabrício M. S. Oliveira, Elizângela A. Rocha, Mainá Bitar, Andrea M. Macedo, Carlos R. Machado, Marcelo V. Caliari, and Glória R. Franco performed data acquisition, analysis, and interpretation of the results; Sílvia R. C. Dias, Mariana Boroni, Mainá Bitar, Fabrício M. S. Oliveira, Marcelo V. Caliari, and Glória R. Franco contributed to drafting the manuscript; Sílvia R. C. Dias, Mariana Boroni, Mainá Bitar, Fabrício M. S. Oliveira, Andrea M. Macedo, Carlos R. Machado, Marcelo V. Caliari, and Glória R. Franco critically revised intellectual content of the work.

ACKNOWLEDGMENTS

This work was supported by CNPq, CAPES/PNPD, and FAPEMIG. The authors would like to thank Dr. Ronaldo Nagem and Dr. Daniela Castanheira Bartholomeu from Universidade Federal de Minas Gerais (UFMG) for giving permission to use the AKTA Prime System and Dr. Ronaldo Nagem for kindly providing the pET28aTEV vector; Dr. Élida Mara Leite Rabelo from UFMG for kindly providing the *S. mansoni* extracts; Moluscário Lobato Paraense (CPqRR) for cercariae donation, Carlos Manoel Afonso for animal care; and Neuza Rodrigues Antunes for laboratory technical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fgene.2014.00174/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 12 February 2014; accepted: 22 May 2014; published online: 11 June 2014.
*Citation: Dias SRC, Boroni M, Rocha EA, Dias TL, de Laet Souza D, Oliveira FMS, Bitar M, Macedo AM, Machado CR, Caliari MV and Franco GR (2014) Evaluation of the Schistosoma mansoni Y-box-binding protein (SMYB1) potential as a vaccine candidate against schistosomiasis. Front. Genet. **5**:174. doi: 10.3389/fgene.2014.00174*
This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Genetics.

frontiers in GENETICS

REVIEW ARTICLE
published: 11 June 2014
doi: 10.3389/fgene.2014.00176

Revisiting glucose uptake and metabolism in schistosomes: new molecular insights for improved schistosomiasis therapies

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A better understanding of the molecular mechanisms required for schistosomes to take up glucose, the major nutritional source exploited by these blood flukes from their mammalian hosts and the subsequent metabolism required to fuel growth and fecundity, can provide new avenues for developing novel interventions for the control of schistosomiasis. This aspect of parasitism is particularly important to paired adult schistosomes, due to their considerable requirements for the energy needed to produce the extensive numbers of eggs laid daily by the female worm. This review describes recent advances in characterizing glucose metabolism in adult schistosomes. Potential intervention targets are discussed within the insulin signaling and glycolysis pathways, both of which play critical roles in the carbohydrate and energy requirements of schistosomes.

Keywords: schistosome, *Schistosoma*, glucose uptake and metabolism, insulin signaling pathway, glycolysis signaling pathway

INTRODUCTION

Schistosomiasis remains one of the most devastating tropical parasitic diseases (Colley et al., 2014), with an estimated 200 million people infected (85% live in Africa), and about 700 million people at risk in 74 countries (Nour, 2010). This is despite the availability of a highly effective drug [praziquantel (PZQ)] and extensive ongoing control programs involving mainly mass drug administration. Important shortcomings of PZQ include its relative inactivity against migratory juveniles and developing worms (Gonnert and Andrews, 1977) and its inability to prevent reinfection. Schistosomes have a complex life cycle, with discrete stages perfectly adapted to their differing host and free-living environments, to promote survival and transmission. The mammalian endoparasitic life cycle begins with the penetration of cercariae, released from freshwater snails, though host skin; then these larvae develop into schistosomula which move to the lungs and pass down the mesenteric vasculature (*S. mansoni*, *S. japonicum*) or urinary bladder venous plexus (*S. haematobium*) where host signals stimulate further development of the juvenile males and females. Pairing and sexual maturation of the adult worms results in extensive egg production. It is the schistosome eggs that are responsible for both the severe pathology associated with schistosomiasis, due to granuloma formation around the ova trapped in tissues, and transmission. The latter is dependent upon the eggs being released from the definitive mammalian host into water and their hatching to release miracidia which penetrate the appropriate freshwater snail intermediate host. Within the haemocoel of the snail, the miracidia form sporocysts, in which further asexual propagation releases larval cercariae. Such processes of multiplication and proliferation are highly energy consuming and schistosomes are entirely reliant on their hosts for the essential nutrients they require for development, reproduction and metabolism.

It is anticipated that better strategies, including vaccines, for schistosomiasis control will rely on an improved understanding of how schistosomes utilize host nutrients, neuro-endocrine hormones and signaling for their survival, development and maturation. Comprehensive deciphering of the available schistosome genomes, transcriptomes, and proteomes is becoming increasingly important for understanding the highly adapted relationship between parasite and hosts on the path to identifying novel drug or vaccine targets (Berriman et al., 2009; *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium, 2009; Young et al., 2012). Resident in the mammalian host bloodstream, schistosomes import essential nutrients across the body surface and through the gut and it is striking that the adult worms consume their dry weight of glucose from the host every 5 h (Bueding, 1950) in order to survive. Here, we focus on the molecular features associated with glucose uptake and metabolism in schistosomes during the intra-mammalian stages of development. We also discuss the essential role these processes play in worm development, growth and maturation, and the potential for the associated molecular components might have as novel vaccine or drug targets.

CLONING AND CHARACTERIZATION OF SCHISTOSOME GLUCOSE TRANSPORTER PROTEINS

It is recognized that glucose is the common currency of cellular metabolism and all cells import glucose across their hydrophobic surface membranes using glucose transporter proteins (GTPs) (Lienhard et al., 1992). Four glucose transporters (SGTP1, 2, 3, and 4) have been identified in *S. mansoni* (Skelly et al., 1994; Krautz-Peterson et al., 2010), of which SGTP1 and SGTP4 display glucose transport activity, and are markedly inhibited by cytochalasin B in *Xenopus* uptake assays (Skelly et al., 1994). *SGTP1* is present in a number of life stages (eggs, sporocysts, cercariae, schistosomula and adult female and male worms) while *SGTP4* has been detected only in the intra-mammalian forms, where it appears after the transformation of the cercariae into schistosomula and the appearance of the double membrane of the adult worm tegument (Skelly and Shoemaker, 1996). Both SGTP1 and SGTP4 are localized to the tegument of adult worms and schistosomula (Skelly and Shoemaker, 1996). SGTP4 seems to be localized uniquely to the apical membranes of the tegument, while SGTP1 is located on the tegumental basal membrane and within the worm body, particularly in muscle. This localization of the two SGTPs in the schistosome tegument implies that the host-interactive SGTP4 protein facilitates the import of glucose from the host bloodstream into the tegument while SGTP1 serves to move free glucose from the surface into the interstitial fluids providing a nutrient source for the internal tissues (Zhong et al., 1995). Probing of cryosections of adult *S. japonicum* with anti-SGTP1 and SGTP4 antibodies showed the same localization for SGTP1 and SGTP4 in the tegument of *S. japonicum* and *S. mansoni* suggesting sequence homology between the SGTPs of the two species (Jiang et al., 1999). Sequencing of the *S. japonicum* genome has indicated this is the case for *SjGTP1* although only partial sequence is available for *SjGTP4* (http://chgc.sh.cn/japonicum/) (Supplementary Table 1). Full length sequences for *GTP1* and *GTP4* have also been identified in *S. haematobium* (Young et al., 2012) (Supplementary Table 1). RNAi studies have shown that *SGTP1*- or *SGTP4*-suppressed schistosomula and adult worms of *S. mansoni* have an impaired ability to transfer glucose (Krautz-Peterson et al., 2010). Worms having both *SGTP1* and *SGTP4* suppressed showed a further decreased capacity to take up glucose compared with worms having only a single *SGTP* gene knocked down. Significantly fewer parasites having *SGTP*-knocked down survived after prolonged culture in glucose-depleted medium compared with controls. Further, *SGTP*-knockdown parasites exhibited less viability *in vivo* after infection of mice. Taken as a whole, these studies emphasized the important roles of SGTP1 and SGTP4 in the transfer into schistosomes of exogenous glucose, which is the major energy source for parasite survival and development in the mammalian host.

In addition, SGTP2 and SGTP3 are also suspected to be associated with glucose uptake in schistosomes. SGTP2 expression was shown to be limited to the female reproductive tract (Skelly et al., 1998) but the protein did not appear to be functional in glucose transport and it was speculated that a deletion mutation in SGTP2 occurred in its recent evolutionary history resulting in its loss of function (Skelly et al., 1994). *In silico* annotation has indicated that SGTP3 (Smp_127200) is the most recently identified component considered as a potential glucose transporter in *S. mansoni* (Krautz-Peterson et al., 2010) but further functional investigation is required to confirm this observation.

SIGNAL TRANSDUCTION REGULATES SCHISTOSOME GLUCOSE IMPORT

When considering GTPs in mammalian cells, it is impossible to ignore the important role of insulin in the dual regulation of increasing glucose uptake and glycogen synthesis (glycogenesis). The *Caenorhabditis elegans* genome encodes a single insulin/IGF-1-like receptor (*daf-2*) and 40 members of insulin-like peptides (*ILPs*) (Li et al., 2003). Recently, it has been confirmed that in *C. elegans,* daf-2 modulates glucose transport via the insulin signaling pathway, in similar manner to that found in mammalian cells (Beall and Pearce, 2002). Whether insulin regulates glucose uptake in schistosomes by a similar mechanism to that observed in *C. elegans* remains to be determined. Schistosomes are unable to synthesize insulin (2009) although insulin-degrading proteases (inferred from annotation) have been identified in *S. japonicum* (Sjp_0009920, http://chgc.sh.cn/japonicum/) and *S. mansoni* (Smp_128100, http://www.genedb.org/Homepage/Smansoni) which may have the same ability to degrade the B chain of host insulin as mammalian cells (Affholter et al., 1988). Microarray analysis demonstrated that adult worms of *S. japonicum* depend on host insulin for growth and fecundity (You et al., 2009). Insulin stimulates glucose uptake, improves the viability of schistosome larvae *in vitro* (Vicogne et al., 2004) and promotes the metabolism and development of adult worms (Saule et al., 2005). These findings contrast with earlier reports which suggested that insulin does not affect glucose consumption by schistosomes*in vitro* (Clemens and Basch, 1989), and that most glucose is imported by adult worms via carrier-mediated diffusion (Skelly et al., 1998). The more recent study by Ahier et al. (2008) has stressed the importance of insulin in modulating glucose uptake in *S. mansoni*. Accordingly, two types of insulin receptors, belonging to the large class of receptor tyrosine kinases (RTKs), have been isolated from *S. mansoni* (SmIR1 and 2) (Khayath et al., 2007) and *S. japonicum* (SjIR1 and 2) (You et al., 2010), both of which are able to bind human insulin. Transcription levels of the *SjIR*s were shown to be up-regulated in mammalian stage parasites (adult worms and schistosomula), further underpinning their involvement in the host-schistosome interaction (You et al., 2010). The SjIRs are also highly expressed in vitelline gland tissue suggesting an important role in supplying nutrients and shell precursors for egg production, the major function of the vitellaria (You et al., 2010). Whereas immunolocalization analysis showed that SmIR1 and SjIR1 are located at the basal membrane of the tegument and in muscles of adult worms (Khayath et al., 2007; You et al., 2010), and have the same location as SGTP1 and SGTP4 (Skelly et al., 1994). While SmIR2 and SjIR2 are, instead, expressed in parenchymal cells of adult males and vitelline cells of females, indicating the two receptor types could have different functions (Khayath et al., 2007). On scrutiny of the recently published *S. haematobium* genomic sequence (Young et al., 2012) by protein BLAST, we identified only *IR2*, although

it is likely that *IR1* is also present. Further information on insulin receptors in the three main clinically relevant schistosome species is presented in Supplementary Table 1.

Notably, in a murine vaccine/challenge model of *S.japonicum*, immunization with the L1 subdomain (which may contain the insulin binding sites) of the SjIR fusion proteins expressed in *E. coli*, resulted in highly significant reductions in fecal eggs (56–67%), stunting of adult worms (12–42%), a reduction in hepatic granuloma density (55%) and a reduction in the numbers of mature intestinal eggs (75%) (You et al., 2012). The depression in the development of mature eggs following SjIR vaccination supports earlier observations that a low level of insulin in host blood might hamper egg passage through the intestinal tissue (Hulstijn et al., 2001).

The development of a safe, stable and effective vaccine based on the ligand domains of SjIR1 and 2 using peptides derived from their primary sequences, which are highly antigenic with the ability to bind human insulin, may be feasible due to their low homology to human IR (HIR). Additionally, the HIR contains two receptor binding sites, which are the L1 domain and the first and second type fibronectin III repeats of the insulin receptor (Whittaker et al., 2008). The identification and characterization of the fibronectin domains in the SjIRs, which have considerably lower homology to HIR—but may be important in changing the conformation of the kinase domain and through inducing signaling transduction after binding with insulin—may act as additional targets for blocking or interrupting the binding between the SjIRs and host insulin.

Insulin signaling is an important metabolic pathway in regulating glucose uptake and glycogen synthesis in mammals. Interrogation of the KEGG pathways assigned to metabolic processes indicates a complete insulin signaling pathway is present in *S. japonicum* (www.chgc.sh.cn/japonicum/sjpathway), comprising 43 genes with high homology with other species. Based on published genome data, the 43 genes involved in the insulin pathway have also been identified in *S. mansoni* (Berriman et al., 2009) and *S. haematobium* (Young et al., 2012) (Supplementary Table 2), further supporting the existence of a complete insulin signaling pathway in schistosomes. The available information strongly supports a similar role for schistosome IRs in downstream signal transduction for regulating glucose uptake as occurs in mammalian cells (**Figure 1**). Both *S. japonicum* and *S. mansoni* IRs have conserved α2β2 structures and structure modeling analysis showed the conserved structure between the SjIRs and HIR, indicating a common predicted binding interaction which occurs in the ligand domain, inducing the same downstream signal transduction in the tyrosine kinase domain (You et al., 2010). Several approaches including two-hybrid analysis, microarray analysis and inhibitor studies *in vitro* (**Figure 1**) have been used to further support the hypothesis that glucose uptake in adult schistosome worms depends on phosphorylation processes that could be modulated by insulin pathways.

Tyrphostin AG1024 is a potent inhibitor of the RTK venus kinase receptors of *S. mansoni* (Ahier et al., 2008; Vanderstraete et al., 2013b). The venus kinase receptors, named because they contain an atypical venus flytrap (VFT) motif in their extracellular domain, usually present in G-protein coupled C class receptors; the catalytic domains of SmIR1 and SmIR2 and venus kinase receptors (SmVKR1 and SmVKR2) are similar (Vanderstraete et al., 2013b). Both receptor types are important for key biological processes in *S. mansoni* including metabolism and reproduction (Vanderstraete et al., 2013a) and the IRs may be essential in regulating glucose uptake (You et al., 2010). Tyrphostin AG1024 was shown to simultaneously cause inhibition of the functional activity of SmIRs and SmVKRs leading to the killing of both immature and adult *S. mansoni in vitro.* This drug may prove useful for the future design of anti-kinase compounds for anti-schistosome chemotherapy, and as an alternative drug to PZQ, which has no effect on immature worms (Vanderstraete et al., 2013b). SmVKR1 and SmVKR2 are directly associated with parasite growth and fecundity in schistosomes; found only in invertebrates, they are activated by amino-acids (Vicogne et al., 2003) and have an intracellular kinase domain similar to that of SmIRs. These receptors are highly expressed in larval *S. mansoni* and in ovaries of females, indicating involvement in development and reproduction (Gouignard et al., 2012). It is noteworthy that RTKs are considered as attractive anticancer drug or vaccine targets (Arora and Scholar, 2005; Kuwai et al., 2008).

Based on their important involvement in regulating the proliferation and differentiation of vitelline cells and egg embryogenesis (Loverde et al., 2009), parasite RTKs, including IRs and VKRs, transforming growth factor (TGF-β) receptors, epidermal growth factor (EGF) receptor and tumor necrosis factor-α (TNF-α) receptor, have considerable potential as novel intervention candidates against schistosomes and other helminths parasites of clinical and veterinary importance (You et al., 2011).

SCHISTOSOME GENES AND GENE PRODUCTS INVOLVED IN GLYCOLYSIS AND GLYCOGENESIS

During their complex life cycle, schistosomes alternate between consuming host glucose and stored glycogen to provide their energy requirements. When a cercaria penetrates the definitive host and transforms into a schistosomulum, the parasite switches rapidly from carbon dioxide production via the Krebs cycle to lactate production using glycolysis; lactate remains the main end product of glucose degradation as schistosomes develop in their mammalian hosts (Tielens, 1994). In tandem, schistosomes shift rapidly from the consumption of stored glycogen to a dependence on host glucose during the transformation phase from free-living cercariae to schistosomula (Skelly et al., 1993). In the mammalian stages, schistosomula have low expression of respiratory enzymes but regain their capacity for aerobic glucose metabolism as they develop to adult worms (Skelly and Shoemaker, 1995). Schistosomes express a range of mRNAs at relatively high levels associated with anaerobic and oxidative glucose metabolism during the transformation from cercaria to adult, re-emphasizing the fact that adult worms possess a significant capacity to generate energy through aerobic metabolism (Skelly et al., 1993). Glycogen synthesis has been shown to be indirectly proportional to the amount of glycogen already present in adult worms, which in turn is proportional to the size of the parasite (Tielens

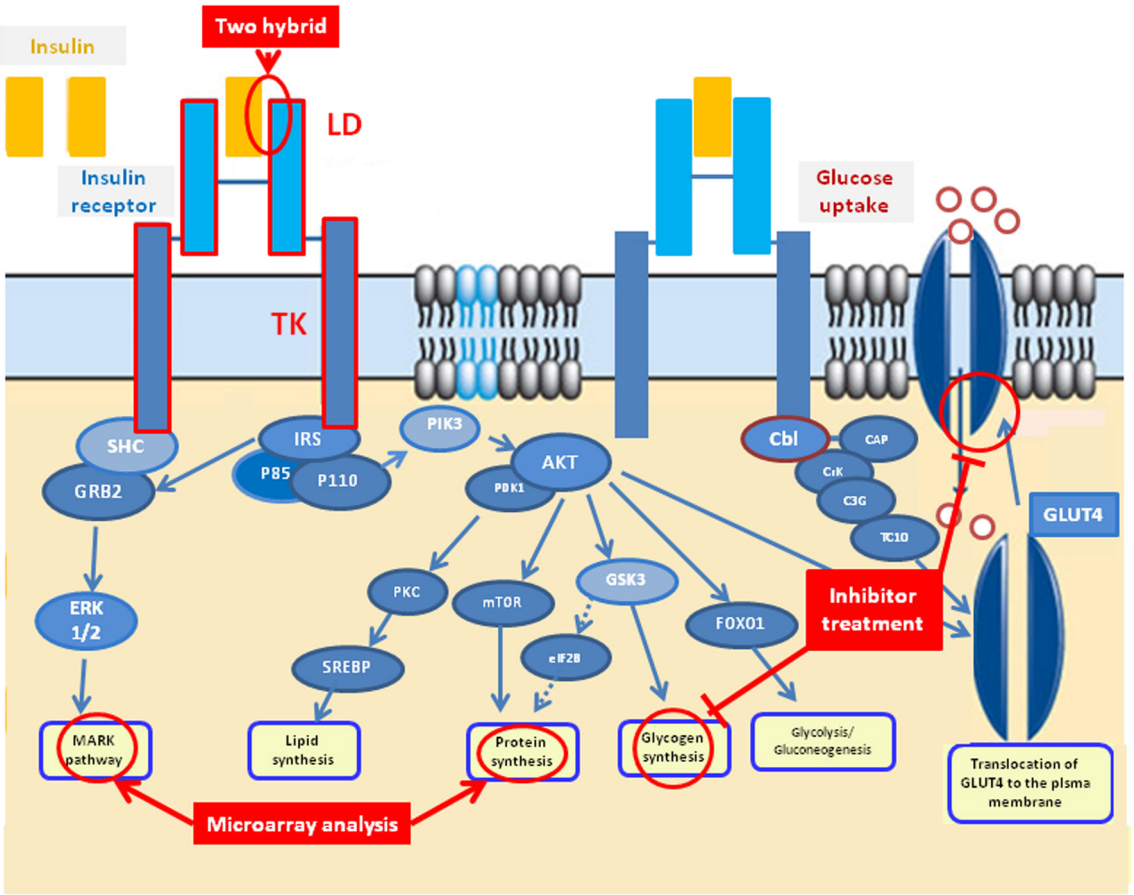

FIGURE 1 | Predicted insulin signaling pathway in schistosomes based on the characterization of schistosome insulin receptors, the published *S. japonicum* KEGG insulin pathway (www.chgc.sh.cn/japonicum/sjpathway) and the human insulin pathway (http://www.google.com/imgres?imgurl=&imgrefurl=http%3A%2F%2Fwww.abcam.com%2Findex.html%3Fpageconfig%3Dresource%26rid%3D10602&h=0&w=0&tbnid=6ZKuhIAsT2VZXM&zoom=1&tbnh=184&tbnw=274&docid=ETqKf7RmabxHnM&tbm=isch&ei=IU2JU6fSDKmysQSx34BQ&ved=0CAIQsCUoAA).

et al., 1990). It appears that glycogen is degraded intermittently for specific purposes such as muscle contraction or tegumental membrane repair, with both functions being more prevalent in adult males (Gobert et al., 2003). Interrogation of the KEGG pathways assigned to metabolic processes indicates a complete glycolytic pathway is present in *S. japonicum* (www.chgc.sh.cn/japonicum/sjpathway), comprising 23 genes with high homology with other species (Supplementary Figure 1; Supplementary Table 3). Mining the published genomic data for *S. mansoni* (Berriman et al., 2009) and *S. haematobium* (Young et al., 2012) shows all 23 genes involved in glycolysis also occur in these two species (Supplementary Table 3).

A number of the key enzymes involved in the glycolytic pathway in schistosomes have been characterized; these include enolase (Ramajo-Hernandez et al., 2007; De La Torre-Escudero et al., 2010), triose-phosphate isomerize (TPI) (Yu et al., 2006; Da'dara et al., 2008), glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (Goudot-Crozel et al., 1989; Charrier-Ferrara et al., 1992), phosphofructokinase (PFK) (Mansour et al., 2000), phosphoglycerate kinase (PGK) (Lee et al., 1995), hexokinase and glucose-6-phosphatise (Kuser et al., 2000). TPI, which converts glyceraldehyde-3-phosphate to dehydroxyacetone phosphate, is a lead anti-schistosome vaccine candidate (McManus and Loukas, 2008), generating an immune response that presumably reduces the capacity of the blood fluke to metabolize glucose via glycolysis for energy production. TPI is present in most cells of schistosome worms and has also been localized on the surface membranes of the newly transformed schistosomulum (Harn et al., 1992), the stage in the mammalian host that is the likely target of an anti-schistosome vaccine. TPI can induce protection against *S. japonicum* challenge in mice (27.9% worm burden reduction) (Zhu et al., 2002), pigs (48% worm reduction) (Zhu et al., 2006) and water buffaloes (48–52% worm reduction) (Yu et al., 2006; Da'dara et al., 2008). Enolase is another key glycolytic/gluconeogenic enzyme that is a physiological receptor of plasminogen, a molecule which is essential for the activation of the host fibrinolytic system, probably to prevent blood clot formation on the schistosome worm surface (Ramajo-Hernandez et al., 2007; De La Torre-Escudero et al., 2010). Disappointingly, the protective efficacy of recombinant, functional enolase in murine vaccine/*S. japonicum* challenge experiments is marginal (Waine et al., 1993).

OTHER COMPONENTS INVOLVED IN GLUCOSE UPTAKE

Acetylcholinesterase (AChE) has been indicated in the modulation of glucose scavenging from mammalian host blood by schistosomes (Camacho and Agnew, 1995). The glucose uptake is regulated by acetylcholine (ACh) interaction with tegumental acetylcholine receptor (nAChR) and AChE. As reviewed by Lee (1996), the secreted AChE may change host cell permeability, have an anti-coagulant role, influence glycogenesis, and play an important function in acetate and choline metabolism as has been shown in nematodes. External AChE may cause the breakdown of local host glycogen stores or block the conversion of glucose to glycogen—as ACh stimulates glycogen synthesis—either of which could make glucose more available in the local environment (Lee, 1996). The function of AChE is supposed to limit the interaction of ACh with its receptor, because inhibition of AChE leads to an effect that mimics ligand excess (Jones et al., 2002). These influences of ACh on glucose uptake can be inhibited through inhibition in turn of either tegumental AChE or tegumental nAChR (Jones et al., 2002). The mammalian stages of schistosomes have AChE and nAChR on their teguments and both components are concentrated on the surface of the adult male, a major surface site for nutrient uptake for the worm pair. The rate of glucose import *in vitro* by *S. haematobium* and *S. bovis* adult worm pairs was shown to be enhanced by approximately 60% at blood (physiological) concentrations of Ach, although *S. mansoni* did not show a similar response (Camacho and Agnew, 1995). It is noteworthy that AChE inhibition in adult worms results in the depletion of tegumental but not muscle glycogen stores; this is relevant since skeletal muscle acts as the primary site for insulin-stimulated glucose disposal in mammals (Thabet et al., 2008). As this observation could not be attributed to the inhibition of glycogen metabolism directly, disruption of sugar transport may have been the cause (Camacho and Agnew, 1995). *In vitro* studies showed that purified polyclonal antibodies against *S. mansoni* AChE were cytotoxic, causing almost total complement-dependent killing of the parasite (Espinoza et al., 1991; Arnon et al., 1999), suggesting AChE may be a highly suitable candidate as a vaccine target, especially as it is highly conserved across a variety of schistosome species (Espinoza et al., 1991) and anti-*S. mansoni* AChE antibodies do not cross-react with human AChE (Espinoza et al., 1991).

As discussed by Hu et al. (2003), cytokines and hormones modulate the integrated response of mammalian hosts to infection by schistosomes. In addition to its essential involvement in the host immune system, an unexpected role for Interleukin-7 (IL-7) has been found in the development, maturation and survival of schistosome worms in mammalian hosts. Infection of mice deficient in IL-7 expression leads to parasite dwarfism (Saule et al., 2002). In this model, IL-7 shares similar, but not identical, effects with the thyroid hormone thyroxin (T4) (Saule et al., 2003). This posed the question as to whether there is a common mediator to their action, which was hypothesized to be host glucose metabolism. Infection with *S. mansoni* resulted in an early peak in glycaemia immediately followed by a peak of insulinemia. In IL-7 + T4 co-treated infected animals, the peak of insulin was abrogated (Saule et al., 2005). The same study further assessed the consequences of experimentally induced glucose- or insulin-level variations on parasite development. Insulin treatment led to increased worm burden and parasite size, thus mimicking the effect of T4 on schistosome development (Saule et al., 2005). Finally, these treatments were also associated with an alteration in the gene expression of schistosome components involved in glucose import (Saule et al., 2005). Overall, IL-7 and T4 regulate schistosome glucose metabolism through modulations in the circulating levels of host glucose and insulin (Saule et al., 2005).

OTHER DRUGS TARGETING GLUCOSE METABOLISM IN SCHISTOSOMES

Artemether is a highly effective anti-malarial drug that also has anti-schistosomal properties. The precise molecular target of the drug in schistosomes remains unproven although there is some evidence that artemether binds to SmSERCA, a putative Ca^{2+}-ATPase of *Schistosoma* (Lepore et al., 2011). Artemether has also been shown to display apparent effects on the carbohydrate metabolism of schistosomes (Zhai et al., 2000b) and key glycolytic enzymes, such as PFK (Xiao et al., 1998) and enolase (Zhai et al., 2000a), any of which might be a target(s) of the drug. Artemether appears to enhance the metabolism of glycogen in adult schistosomes and the inhibition of lactate dehydrogenase thereby reducing the formation of lactate (Xiao et al., 1999). The artemether-induced glycogen decrease in schistosomes was shown to be associated with the inhibition of glycolysis rather than an interference with glucose import (Xiao et al., 1997). Schistosomes recovered from artemether treated animals retain increased glycogen phosphorylase activity, but decreased glucose uptake, due to their decreased glycogen content compared with worms from untreated animals (Shuhua et al., 2000).

Another antimalarial drug mefloquine has shown promise as an antischistosomal agent killing adult schistosomes as well as schistosomula (Manneck et al., 2012). A single dose of mefloquine administered orally to mice infected with *S. mansoni* or *S. japonicum* led to significant reductions in adults and young developing worm burdens of both schistosome species (Keiser et al., 2009). Mefloquine affinity chromatography of crude extracts of *S. mansoni* schistosomula identified one specific mefloquine-binding protein which was the glycolytic enzyme enolase (Manneck et al., 2012). This study also showed that mefloquine and a specific enolase inhibitor—sodium fluoride inhibited enolase activity in crude extracts of schistosomula, although activity of a recombinant form of enolase was unaffected (Manneck et al., 2012). Using isothermal microcalorimetry, the functional inhibition of mefloquine and three known glycolytic pathway inhibitors in schistosomes (sodium fluoride, 3-bromopyruvate, and menadione) were investigated in *S. mansoni* in the presence or absence of glucose. The result suggested a potential role for mefloquine as an inhibitor of glycolysis in lifecycle stages where other targets such as haem degradation are not pertinent (Manneck et al., 2012). Consideration could be given to determine whether mefloquine treatment would be of value in patients who have mixed malaria and *Schistosoma* infections as such coinfections are not uncommon, particularly in Africa, although a concern would be that this strategy might select for *Plasmodium* parasites resistant to mefloquine. Mefloquine monotherapy for schistosomiasis may prove superior

to PZQ alone, since both juvenile and mature schistosome worms would be targeted in an infection. Mefloquine, in combination with PZQ, whether as a full or half dose regimen, can substantially reduce the course of infection, thereby further confirming its potential as an anti-schistosomal drug (Nashwa and Abdel-Fattah, 2011). Both artemether and mefloquine have shown promising anti-schistosomal features against adult and juvenile*S. mansoni* in both T cell-deficient mice and in relatively infected age- and sex-matched immunologically intact control mice (Keiser et al., 2010). Artemether treatment reduced total worm burden ranging between 71.1 and 85.3%, while mefloquine induced reductions of total worm number between 80.4 and 97.8% in athymic and immunocompetent NMRI mice. These results suggest that artemether and mefloquine act T-cell independently and that no synergy with the immune response occurs (Keiser et al., 2010). Notably, mefloquine (Lariam) can produce severe neuropsychiatric and psychiatric side effects, which can cause mental health issues (http://www.fda.gov/downloads/Drugs/DrugSafety/ucm088616.pdf), which would be an important consideration for its use in future as an anti-schistosome therapy.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Glucose metabolism leading to ATP synthesis is critical for the survival of schistosomes. Directly or indirectly interrupting or blocking glucose uptake may represent a realistic strategy for drug or vaccine development as it would likely lead to the starvation of worms and an insufficient supply of energy for growth, pairing, maturation, and fecundity.

However, this may not be a simple undertaking as many different components participate in glucose uptake so the blocking or inhibiting of one gene or gene product may stimulate the schistosome worm to compensate by switching to a sub-pathway or another related pathway, so as to allow the acquisition of glucose. One logical approach may be to design multivalent vaccines or drugs targeting two or more key genes that would depress worm growth, reduce worm burden and fecal egg output, with the simultaneous reduction in hepatic egg-associated disease pathology. This strategy could first be developed and tested in the form of a highly efficacious veterinary-based multivalent transmission blocking vaccine (McManus and Loukas, 2008) for application in animal reservoirs of *S. japonicum* in China and the Philippines, and then extended to target the African schistosomes, *S. mansoni*, and *S. haematobium*, with appropriately designed human clinical trials.

ACKNOWLEDGMENTS

Our work on schistosomiasis is supported by the National Health and Medical Research Council of Australia (ID 496600; ID613671; ID1037304). Donald P. McManus is a NHMRC Senior Principal Research Fellow and Senior Scientist at QIMR Berghofer Medical Research Institute.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fgene.2014.00176/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 March 2014; paper pending published: 02 May 2014; accepted: 23 May 2014; published online: 11 June 2014.

*Citation: You H, Stephenson RJ, Gobert GN and McManus DP (2014) Revisiting glucose uptake and metabolism in schistosomes: new molecular insights for improved schistosomiasis therapies. Front. Genet. **5**:176. doi: 10.3389/fgene.2014.00176*

This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Genetics.

frontiers in GENETICS

ORIGINAL RESEARCH ARTICLE
published: 10 June 2014
doi: 10.3389/fgene.2014.00170

Gonad RNA-specific qRT-PCR analyses identify genes with potential functions in schistosome reproduction such as SmFz1 and SmFGFRs

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In the search for new strategies to fight schistosomiasis, the unique reproductive biology of *Schistosoma mansoni* has come into the focus of research. The development of the gonads and the ability of egg production are fundamental not only for continuing the life cycle but also for pathogenicity. Previous studies of schistosome biology demonstrated an influence of pairing on gonad development of the female and on gene expression profiles in both genders. Due to the limited access to specific tissues, however, most of these studies were done at the level of whole worms neglecting individual tissues that may be targets of pairing-dependent processes. Recently, we established a protocol allowing the isolation of testes and ovaries from adult *S. mansoni*. Here, we describe tissue-specific qRT-PCR analyses comparing transcript levels of selected genes on the basis of RNA from gonads and whole worms. Gene expression in ovary and testes was in some cases found to be significantly influenced by pairing, which was not traceable in whole worms. Among the candidate genes identified as regulated by pairing in gonads were the frizzled homolog SmFz1 and the two fibroblast growth factor receptor homologs SmFGFR-A and SmFGFR-B. First functional characterizations were done, including comparative qRT-PCR analyses, *in situ*-localization experiments, heterologous expression in *Xenopus* oocytes (SmFGFR-A/B), and inhibitor studies using the Fz/Dvl-pathway inhibitor 3289-8625, or BIBF1120 blocking FGFR-signaling. Besides confirming gonad localization and receptor functions, inhibitor-induced phenotypes were observed *in vitro* such as decreased egg production as well as drastic effects on gonad differentiation, morphology, embryogenesis, and survival of adult worms. In summary, these results emphasise the usefulness of tissue-specific qRT-PCRs for selection of candidate genes with important roles in reproduction, allowing subsequent studies to determine their suitability as drug targets.

Keywords: schistosomiasis, *Schistosoma mansoni*, development, helminth reproduction, gonad, frizzled (Fz), fibroblast growth factor receptor (FGFR), organ isolation

INTRODUCTION

Besides their medical importance for humans and animals causing schistosomiasis (bilharzia), schistosome parasites exhibit a number of unusual features. Schistosomes possess a complex life-cycle, they belong to the trematodes but have evolved separate sexes, and their reproductive biology is governed by pairing-dependent processes (Popiel and Basch, 1984; Kunz, 2001; Chitsulo et al., 2004; King et al., 2005). In this context, a unique phenomenon of schistosome biology is that the female requires permanent pairing-contact with the male to become sexually mature. Without a pairing partner, the female remains sexually immature, containing stem cell-like precursor cells in the vitellarium and an ovary filled with immature oogonia. Following pairing, differentiation processes are induced leading to the complete differentiation of the vitellarium and the ovary, which contain mature S4-vitellocytes and primary oocytes, respectively. Both are needed for the formation of composite trematode eggs (Shaw and Erasmus, 1982; Popiel and Basch, 1984; Kunz, 2001). This is controlled by signal transduction processes and paralleled by a remarkable increase of the body size of the female (Knobloch et al., 2007; LoVerde et al., 2009; Beckmann et al., 2010). An improved understanding of the reproductive biology of this exceptional parasite is necessary, as the eggs are responsible for the pathology of schistosomiasis.

Since there is increasing fear of the development of resistance against the only commonly used drug applied to fight all schistosome species, Praziquantel (PZQ), and because there is no vaccine available yet, great efforts are being made to search for alternatives (Fenwick and Webster, 2006; Doenhoff et al., 2008; Melman et al., 2009). Among these are genomic, transcriptomic, and proteomic studies that have provided a huge amount of valuable information about the genetic repertoire of schistosomes

(Verjovski-Almeida et al., 2003; Liu et al., 2006; Hokke et al., 2007; Berriman et al., 2009; Schistosoma japonicum Genome Sequencing and Functional Analysis Consortium, 2009; Protasio et al., 2012; Wilson, 2012; Young et al., 2012).

Now, in the dawn of the post-genomic era, methods are required to make use of the available genome data and to functionally characterize genes of interest. To this end we have established a protocol for the isolation of pure, intact testes and ovaries from adult schistosomes (Hahnel et al., 2013). Among other outcomes, it was demonstrated that gonad-specific RNA of high quality was obtained from each of these tissues allowing a detailed characterization of gene expression at the tissue level. In parallel, data on G protein-coupled receptors (GPCRs) were obtained (Zamanian et al., 2011; Hahnel et al., unpublished), and the influence of pairing on males was investigated by a combinatory transcriptomics approach using SuperSAGE and microarray hybridization (Leutner et al., 2013). These studies provided hints toward new genes that are influenced by pairing. Among these were gene homologs of the frizzled receptor SmFz1 (Smp_118970/173940), the membrane progestin receptor component 1 SmPMRC1 (Smp_093700), the RNA binding protein Musashi (Smp_157750), and the transmembrane receptor Notch (Smp_050520). Finally, a recent study identified neoblast-like stem cells embedded in somatic tissue in adult *S. mansoni*, and evidence was obtained for a role of a fibroblast growth factor receptor homolog, SmFGFR-A (Smp_175590), for the maintenance of these neoblast-like cells (Collins et al., 2013). Since the latter study provided additional evidence for SmFGFR-A transcripts in testes, this gene and a second member of this gene family in *S. mansoni*, SmFGFR-B (Smp_157300), were included in the following analysis.

To investigate the influence of pairing on the transcription of some of the above mentioned genes at the level of the gonads we performed comparative qRT-PCR analyses. Evidence was obtained that this approach allows the discovery of a pairing-influence on the transcription of some of these genes in the gonads, which is not detectable at the whole worm level. Furthermore, first functional characterization of SmFz1 and SmFGFR-A/B by molecular, biochemical, physiological, and morphological analyses confirmed their potential roles in gonad differentiation and reproduction. Based on the fatal phenotypes observed, our results using inhibitors against the Wnt/Fz-pathway (3289-8625) or against FGFR-signaling (BIBF1120) additionally identified both as interesting lead compounds. Here we show that the combination of organ isolation and detailed tissue-specific expression analyses offer new avenues for the identification and characterization of potential new drug targets, which are desperately needed in the fight against schistosomiasis.

MATERIALS AND METHODS

ETHICS STATEMENT

All animal experiments were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (ETS No 123; revised Appendix A) and have been approved by the Regional Council (Regierungspraesidium) Giessen (V54-19 c 20/15 c GI 18/10).

PARASITE MAINTENANCE

A Liberian strain of *Schistosoma mansoni* was maintained in *Biomphalaria glabrata* and in Syrian hamsters (*Mesocricetus auratus*) (Grevelding, 1995). Adult worms were obtained by hepatoportal perfusion of hamsters at day 42 post-infection. Unisexual worm populations were generated by monomiracidial intermediate-host infection as described elsewhere (Grevelding, 1995). Adult worms were transferred to Petri dishes of 60 mm diameter size containing 4 ml M199 medium (Sigma-Aldrich; supplemented with 10% Newborn Calf Serum (NCS), 1% HEPES [1 M] and 1% ABAM-solution [10,000 units penicillin, 10 mg streptomycin and 25 mg amphotericin B per ml]) in groups of either 20 couples, or 25 males, or 50 females per Petri dish and kept *in vitro* at 37°C and 5% CO_2.

ISOLATION OF RNA AND qRT-PCR ANALYSES

Testes and ovaries of adult worms from bisexual and unisexual infections were isolated by a combined detergent/enzymatic-based approach as described in detail by Hahnel et al. (2013). Total RNA from adult schistosomes and gonad tissue was extracted using the PeqGOLD TriFast reagent (Peqlab) following the manufacturer's protocol. For this, 5–10 adult worms or 50–100 testes and ovaries, respectively, were incubated in 500 µl TriFast-solution. Pairs were separated by repeated pipetting immediately before processing. The adult worms were mechanically homogenized with a plastic piston whereas gonads were frozen in liquid nitrogen and thawed on ice three times to enhance tissue disintegration. Precipitation of total RNA in 2-propanol was aided by addition of 35 µg glycogen (RNase-free PeqGOLD glycogen, Peqlab). RNA quality and quantity were checked by electropherogram analysis employing the BioAnalyzer 2100 (Agilent Technologies). In brief, 1 µl of resuspended RNA was loaded on an Agilent RNA6000 Nano Chip according to the manufacturer's instructions and analyzed using the device setting "EukaryoteTotal RNA Nanoassay."

Synthesis of cDNA was performed using the QuantiTect Reverse Transcription Kit (Qiagen) comprising a genomic DNA wipe out step and 500 ng of total RNA per reaction. The obtained cDNA was diluted 1:20 and used in subsequent qRT-PCR analyses. The detection of synthesized DNA double strands was based on the incorporation of SYBRGreen using PerfeCTa SYBR Green Super Mix (Quanta). To distinguish between specific amplification products and unspecific primer dimers following each qRT-PCR analysis, a melting point analysis was carried out. Primer 3 Plus software was used for primer design, and the amplification products had sizes between 140 and 160 bp. Primers were designed to have melting points at 60°C (Table S1) and were commercially synthesized (Biolegio, Netherlands). Amplification reactions were done in triplicate, and analyses were performed using a relative quantification against the reference gene actin (Smp_161930) with the $\Delta\Delta$Ct method (Livak and Schmittgen, 2001).

GERMINAL VESICLE BREAKDOWN (GVBD) ASSAYS IN *XENOPUS* OOCYTES

The tyrosine kinase domains of SmFGFR-A and SmFGFR-B were amplified by PCR using cDNA from adult worms as template.

For the PCR reactions the following primers were used: 5′FGFR-B_TK-*Bam*HI: GGA TCC ATG AAA TGG TAT CTT CAG AGA GTC AAC AGC, 3′FGFR-B_TK-*Xba*I: TCT AGA TTC ACT AGT TTC AGT ACG ACC ATC and 5′FGFR-A_TK-*Bam*HI: GGA TCC GAA ATG GTT CAA CCA TCC AAA TAT TTT CCA CAG, 3′FGFR-A_TK-*Xho*I: CTC GAG TCC TTC AGG TCA CCA TAA CTG. All primers contained restriction sites at their 5′-end as indicated to allow an insertion of amplification products into the vector pcDNA3.1-B. The correct ORFs of the kinase constructs were confirmed by sequencing (LGC Genomics, Berlin). Plasmids were linearized by the restriction enzyme *Pme*I. Capped messengerRNA (cRNA) encoding the different TK domains were synthesized *in vitro* using the T7 mMessage mMachine kit (Ambion) and analyzed as described previously (Long et al., 2010). cRNA preparations were microinjected into *Xenopus laevis* stage VI oocytes according to a standard protocol (Vicogne et al., 2004). Each oocyte was injected with 60 ng of cRNA in the equatorial region and incubated at 19°C in ND96 medium. After 18 h, GVBD was detected by the appearance of a white spot at the center of the animal pole. For kinase inhibitor studies sets of 10 oocytes were freshly injected with SmFGFR-A or SmFGFR-B kinase domain constructs and placed in ND96 containing different concentrations of BIBF1120 (Vargatef, CAS-No. 656247-17-5; SelleckChem, dissolved in DMSO). GVBD was observed after 18 h. Non-injected oocytes served as negative controls. For positive controls, the natural hormonal stimulus progesterone was used (Sadler and Maller, 1983).

Dead kinase variants of SmFGFR-A and SmFGFR-B kinase domains (SmFGFR-A_TK-ko and SmFGFR-B_TK-ko) were generated by site-directed mutagenesis. Using the primer combination 5′FGFR-A_TK-ko: GGA TTT GTT GCA AAA TTA TGC GAT AAC GCT TAT GCA TGT ACC CAA GAG G/3′FGFR-A_K-ko: CCT CTT GGG TAC ATG CAT AAG CGT TAT CGC ATA ATT TTG CAA CAA ATC C and 5′FGFR-B_TK-ko: GGT AAA CAC TAT AAA TTA AAA ATT GCT GAT AAT GCA CTT ACA AGA TTT GCT GAA/3′FGFR-B_TK-ko: CCT CTT GGG TAC ATG CAT AAG CGT TAT CGC ATA ATT TTG CAA CAA ATC C, the Mg^{2+}-binding motif DFG of the kinase domains was changed into a DNA motif, as described previously (Vicogne et al., 2004). In addition a constitutively active mutant of the FGFR-B_TK domain was produced by exchanging the lysine within the amino acid motif $YYRK^{519}$ to a negatively charge glutamate ($YYRE^{519}$). This mutation is consistent with the $YYKE^{650}$ variation of the human FGF Receptor 3 (hFGFR3), which led to its ligand-independent activation (Neilson and Friesel, 1996; Webster et al., 1996). For the synthesis of FGFR-B_TK-active the following primer combination was used, 5′FGFR-B_TK-active: AGA TTT GCT GAA AAT TAT TAT CGT GAA ATG AAA AAT GGT CGT GTT CCG/3′FGFR-B_TK-active: CGG AAC ACG ACC ATT TTT CAT TTC ACG ATA ATA ATT TTC AGC AAA TCT.

IN SITU-HYBRIDIZATION EXPERIMENTS

In situ-hybridizations were performed as described elsewhere (Quack et al., 2009; Buro et al., 2013). In short, adult worm pairs were fixed in Bouin's solution (picric acid/acetic acid/formaldehyde; 15:1:5, Sigma Aldrich) before embedding in paraplast (Histowax, Reichert-Jung). Sections of 5 μm were generated and incubated in xylol. Following rehydration, proteins were removed by proteinase K treatment (final concentration 1 μg/ml), and the sections were dehydrated. For hybridization, gene-specific transcripts were synthesized *in vitro* using T7 promoter-containing PCR products, and labeled with digoxigenin following the manufacturer's instructions (Roche Applied Science). For this approach the following primer pairs were used: SmFz1 (5′SmFz1_C-Term: GTG GTA AAA CGC TTG TAT CAT GG/ 3′SmFz1_C-Term: GTA AGC CTA GAC CAG AAT TAG C), SmFGFR-A (5′SmFGFR-A_insitu: GAT GAT GCA ATT AGA CAA CAA AGA G/ 3′FGFR-A_insitu: CGA TTA TCG GGA TCT TGT GAC), SmFGFR-B (5′SmFGFR-B_insitu: GTA TCT TCA GAG AGT CAA CAG C/ 3′SmFGFR-B_insitu: CGA TGA CGA CGC AGA TAC TC). To allow the synthesis of antisense or sense transcripts one primer of each reaction was tagged with the T7-sequence at its 5′-end. Labeled sense and antisense transcripts of SmFz1 (459 nt), SmFGFR-A (470 nt), and SmFGFR-B (437 nt) were size-controlled by gel electrophoresis. To prove their quality, transcript blots were made to confirm digoxigenin incorporation by alkaline phosphatase-conjugated anti-digoxigenin antibodies, naphthol-AS-phosphate, and Fast Red TR (Sigma Aldrich). All *in situ*-hybridizations were performed for 16 h at 42°C. Sections were stringently washed up to 0.5 × SSC, and detection was achieved as described for transcript blots.

INHIBITOR TREATMENT OF ADULT SCHISTOSOMES

For *in vitro* culture experiments with inhibitors schistosome couples were transferred into supplemented M199 medium 24 h after perfusion. The inhibitors 3289-8625 (Dvl-PDZ Domain Inhibitor II, CAS-No. 294891-81-9; Merck Millipore) and BIBF1120 (Vargatef, CAS-No. 656247-17-5; SelleckChem) were dissolved in dimethyl sulfoxide (DMSO) and added in various concentrations to the culture medium. Control groups were cultured in medium containing DMSO only. Couples were kept *in vitro* at 37°C and 5% CO_2, and medium and additives were refreshed daily.

MORPHOLOGICAL ANALYSES

Egg development and worm morphology were monitored by bright-field microscopy (CX21, Olympus; Labovert FS, Leitz), and images were acquired by a digital camera (SC30, Olympus) with CellSens Dimension software (Olympus).

For further morphological analyses, adult worms were fixed for at least 24 h in AFA (ethanol 95%, formaldehyde 3%, and glacial acetic acid 2%), stained for 30 min with 2.5% hydrochloric carmine (CertistainH, Merck), and destained in acidic 70% ethanol. After dehydration in 70, 90, and 100% ethanol for 5 min each, worms were preserved as whole-mounts in Canada balsam (Merck) on glass slides (Neves et al., 2005). CLSM images were made on a Leica TSC SP2 microscope using a 488 nm He/Ne laser and a 470 nm long-pass filter in reflection mode as described before (Beckmann et al., 2010).

EdU-INCORPORATION ASSAY

S. mansoni couples were treated with 5 μM of BIBF1120 or DMSO only for at least 48 h *in vitro*. After the first 24 h the medium was additionally supplied with 10 μM of thymidine analog 5-ethynyl-2′-deoxyuridine (EdU). EdU incorporation into

adult schistosomes was detected essentially according to the manufacturer's instructions (Click-iT EdU Imaging Kit; Molecular Probes, Darmstadt, Germany), and as described by Collins et al. (2013). Briefly, treated schistosome couples were separated and subsequently fixed for 6 h in 4% paraformaldehyde in PBSTx (PBS with 0.3% Triton X-100). The worms were then rinsed once in PBSTx, before dehydration in 50% MeOH in PBSTx for 10 min at room temperature (RT) with shaking, followed by a 10 min in 100% MeOH. Parasites were stored in fresh MeOH at −20°C.

The worms were rehydrated through one 10 min wash each in 50% MeOH in PBSTx then PBSTx, after which they were treated with 6 μg/ml proteinase K for 25 mins at RT. The samples were post-fixed in 4% formaldehyde in PBSTx for 10 min at RT. After two washes in 3% BSA in PBS, the worms were incubated in Alexafluor 647 Click-iT reagent for 30 min with shaking. From this point on the samples were protected from light. Following the Click-iT reaction, the worms were washed twice in 3% BSA in PBS. Parasites were counterstained overnight at 4°C with 8 μM Hoechst 33342 in PBSTx. They were washed twice in PBSTx for 15 min at RT before being mounted on slides with Rotimount Fluorcare (Carl Roth, Karlsruhe, Germany). The specimens were viewed on a Leica TCS SP2 confocal microscope. Hoechst was excited with a 405 nm laser, and Alexafluor 647 was excited at 633 nm.

IN SILICO ANALYSES

The following public domain tools were used: Gene DB (http://www.genedb.org/Homepage), Welcome Trust Sanger Institute S. mansoni OmniBlast (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/smansoni/omni), NCBI BLAST (http://blast.ncbi.nlm.nih.gov/), SMART (http://smart.embl-heidelberg.de/). Primer 3 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

RESULTS

INFLUENCE OF PAIRING CONTACT ON GENE TRANSCRIPTION IN THE GONADS

In order to address the question whether the influence of pairing on transcription cannot only be seen at the whole worm level, but also in isolated gonads, we performed qRT-PCR analyses. To this end we compared relative transcript levels of selected genes between pairing-unexperienced (um) and pairing-experienced (em) *S. mansoni* males and females (uf/ef) as well as isolated testes and ovaries from such worms obtained using the recently established organ isolation protocol (Hahnel et al., 2013).

Most of the analyzed genes showed an up-regulation of transcription upon pairing at the levels of whole male worms (**Figure 1A**). This included genes encoding the two FGFR homologs SmFGFR-A and SmFGFR-B as well as a homolog of the progestin membrane receptor component 1 SmPMRC1 (Smp_093700), and a homolog of the RNA-binding protein Musashi (Smp_157750). In contrast, transcription rates of a Notch receptor homolog (Smp_050520) and the frizzled receptor SmFz1 seemed not to be affected by pairing. Focusing on the male gonads, however, all analyzed genes were transcribed more abundantly in the testes of em compared to those of um. With respect to the testes, especially SmFz1, the Notch homolog, and Musashi showed the highest up-regulation in their transcript levels upon pairing contact. These findings revealed pairing-dependent differences in transcriptional activity in the gonads which were not apparent at the whole-worm level.

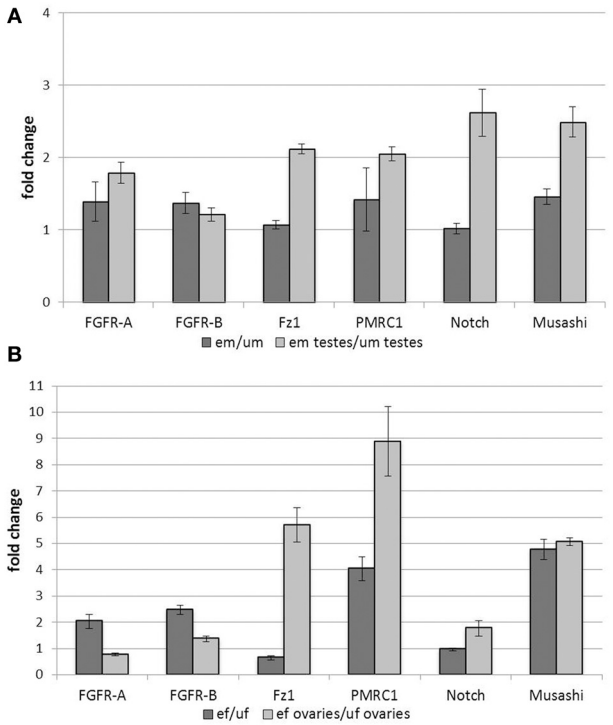

FIGURE 1 | Influence of pairing on gene transcription in adult worms and gonads. Analyses of the influence of pairing on the transcription rates of candidate genes in whole worms and gonads. Transcription of SmFGFR-A, SmFGFR-B, SmPMRC1, and the musashi homolog were up-regulated in em compared to um, whereas SmFz1 and Notch were not differentially transcribed at the whole worm level. In contrast, focusing on the testes, all analyzed genes were transcribed more abundantly in the testes of em compared to those of um **(A)**. Comparably, in female worms, most of the candidate genes were up-regulated following pairing with the exception of Notch and SmFz1, which appeared not differentially and less transcribed in ef compared to uf, respectively. Focusing on ovary-specific gene expression, transcription of SmPMRC1, Musashi as well as SmFz1 were strongly up-regulated by pairing. Compared to this, pairing led to a less remarkable effect on the expression the two FGFR-homologs and Notch in the female gonad **(B)**. In both diagrams the statistical evaluation of three technical replicates is shown (error bars indicated).

In female worms, most of the genes investigated were up-regulated by pairing with the exception of SmFz1 and Notch, which seemed to be slightly down-regulated or non-regulated, respectively (**Figure 1B**). Again, tissue-specific analyses revealed paring-dependent transcription regulation in the ovary, which was not always apparent at the whole worm level. Besides SmPMRC1 and musashi, which were both also strongly influenced by pairing in the ovary, SmFz1 was found to be strongly up-regulated in this organ. Compared to this, pairing led to a less remarkable effect on the expression of the two FGFR-homologs and Notch in the female gonad.

SEQUENCE ANALYSES OF SmFz1, SmFGFR-A AND SmFGFR-B

Searching the genome of *S. mansoni* for frizzled genes using the database GeneDB (Protasio et al., 2012) an annotated entry (Smp_118970) was found coding for the C-terminal fragment of a frizzled homolog named SmFz1. Full-length cloning of SmFz1 revealed that the missing N-terminal part was coded by another sequence annotated as Smp_173940. The whole open reading frame (ORF) of the frizzled gene consists of 2091 bp and an alignment with the GeneDB entries Smp_119870/Smp_173940 revealed a sequence identity of 99% (accession number: KJ820759).

Sequence analyses showed that the predicted protein sequence of SmFz1 contained typical features of frizzled receptors, which belong to the superfamily of seven transmembrane receptors (Schiöth and Fredriksson, 2005) (**Figure 2**). A signal peptide of 18 amino acids length is located at the N-terminus (M^1 to C^{18}), which is probably involved in the localization of the receptor to the plasma membrane. This motif is followed by an extracellular cysteine-rich domain (CRD) (K^{33} to K^{147}) sufficient for the binding of Wnt ligands (Schulte, 2010). The frizzled transmembrane domain extends from L^{193} to R^{560} and contains seven transmembrane helices (THMs). The seventh TMH is followed by the amino acid motif K^{545}TLVSW. This corresponds to the conserved Disheveled (Dvl) binding motif consensus sequence KTXXXW located at the beginning of the intracellular C-terminus of frizzled receptors (Wong et al., 2003).

The genome of *S. mansoni* encodes two FGFR homologs named SmFGFR-A (Smp_175590) and SmFGFR-B (Smp_157300). A detailed *in silico* analysis showed that SmFGFR-A consists of 918 amino acids (**Figure 2**). The extracellular N-terminus starts with a signal peptide sequence (M^1–G^{26}) followed by two extracellular immunoglobulin(IG)-like domains located at the positions T^{39} to S^{94} and S^{310} to M^{403}. IG-like domains are common features of FGFRs and are involved in ligand binding and auto-regulation processes (Eswarakumar et al., 2005). The single TMH is located from position P^{417} to I^{439}. The intracellular C-terminus contains a catalytic tyrosine kinase (TK) domain (F^{582} to I^{880}), which is involved in downstream signaling events (Eswarakumar et al., 2005).

In contrast to SmFGFR-A, the N-terminal signal peptide as well as characteristic IG-like domains are missing within the SmFGFR-B protein sequence (643 aa) (**Figure 2**). At position P^{101} to N^{189} a putative IG-like domain was identified, but the degree of homology was not significant according to SMART. The single TMH is located at position W^{199} to W^{221} followed by the catalytic tyrosine kinase domain (L^{353} to L^{617}).

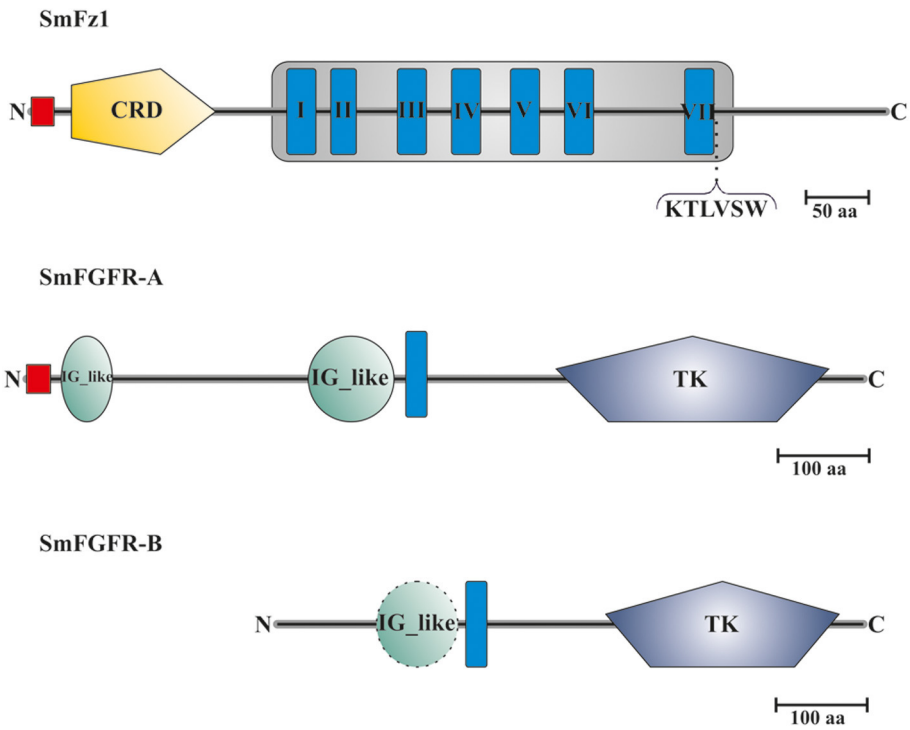

FIGURE 2 | Domain structure analyses of SmFz1, SmFGFR-A, and SmFGFR-B. Schematic structures of the frizzled homolog SmFz1, and the two FGFR homologs SmFGFR-A and SmFGFR-B from *S. mansoni*. SmFz1 comprises an N-terminal signal peptide sequence (red) and an extracellular CRD domain (yellow) sufficient for Wnt ligand binding. The frizzled transmembrane domain (gray box) contains seven TMHs (I–VII). Following TMH VII, a Dvl-binding motif (KTLVSW) is located at the beginning of the intracellular C-terminus. Both FGFRs possess TK domains within their intracellular C-termini but differ in the structures of their extracellular parts (TMHs in blue). SmFGFR-A consists of an N-terminal signal peptide sequence (red) followed by two IG-like domains (green) sufficient for ligand binding **(A)**. In contrast the N-terminus of SmFGFR-B is smaller in size and contains only one putative IG-like domain (dashed line), which was not rated as significant **(B)**.

LOCALIZATION OF SmFz1, SmFGFR-A AND SmFGFR-B TRANSCRIPTS BY *IN SITU*-HYBRIDIZATION

To localize transcripts of SmFz1 and the FGFR homologs SmFGFR-A and SmFGFR-B in schistosomes *in situ*-hybridization experiments were performed on sections of *S. mansoni* couples (**Figure 3**). As predicted by the gonad-specific qRT-PCR experiments, the occurrence of transcripts of all three transmembrane receptors was confirmed in the testes of males and in the ovaries of females. In addition, SmFGFR-B transcripts were detected in the vitellarium of the female and in the gastrodermis as well as the parenchyma of both genders. For SmFGFR-A weak signals also occurred in the parenchyma (data not shown), which is in accordance with former whole mount *in situ* hybridization studies (Collins et al., 2013).

BLOCKING OF FRIZZLED-DVL SIGNALING LED TO DRAMATIC PHENOTYPES IN THE GONADS AND INHIBITED EGG EMBRYOGENESIS

To investigate the role of Frizzled-signaling in reproduction and developmental processes of schistosomes, *S. mansoni* couples were treated *in vitro* with different concentrations of the commercially available compound 3289-8625 (also Dvl-PDZ domain inhibitor II, Merck Millipore). This inhibitor blocks canonical Frizzled-signaling by binding to the PDZ (post-synaptic density-95/discs large/zonula occludens-1) domain of the Frizzled downstream interaction partner Disheveled (Dvl) with an IC_{50} of 12.5 µM in cell culture experiments (Grandy et al., 2009; Voronkov and Krauss, 2013). Because of the drug's specificity targeting the Frizzled-Dvl interaction directly we performed database analyzes to identify Dvl homologs in the genome of

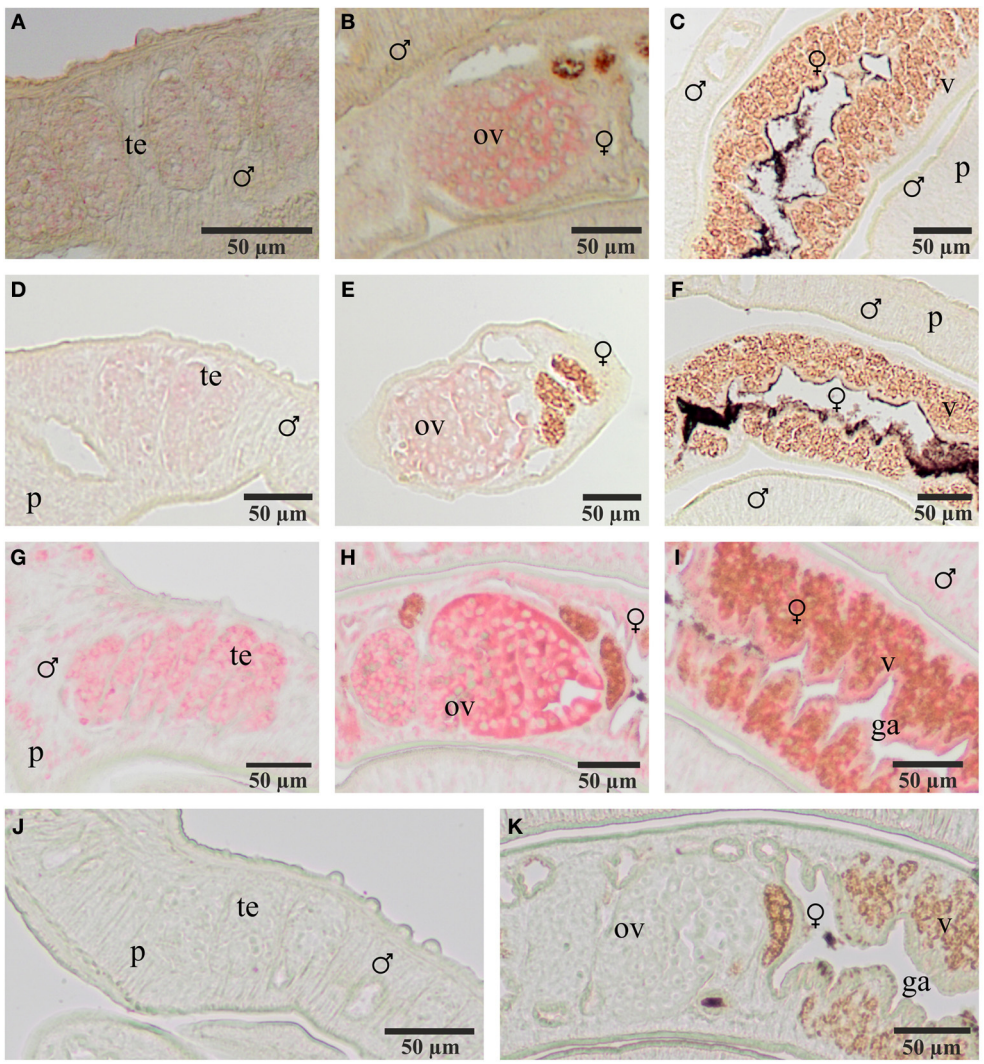

FIGURE 3 | Localization of transcripts of SmFz1, SmFGFR-A, and SmFGFR-B in adult *S. mansoni*. Results of *in situ*-hybridization experiments to localize transcripts of SmFz1 **(A–C)**, SmFGFR-A **(D–F)**, and SmFGFR-B **(G–I)** using DIG-labeled antisense-RNA probes on 5 µm sections of *S. mansoni* couples. Transcripts of all genes were detected in the testes of the male and the ovary of the female. SmFGFR-B transcripts were also observed in the vitellarium of the female and the parenchyma as well as the gastrodermis of both genders. Negative controls using sense-RNA probes showed no color reaction **(J,K)**. [te, testes; ov, ovary; v, vitellarium; p, parenchyma, g, gastrodermis; ♂, male; ♀, female].

S. mansoni. As results two Dvl genes SmDvl1 (Smp_162410) and SmDvl2 (Smp_020300) were found and their transcription in testes and ovaries was confirmed by gonad-specific RT-PCRs (data not shown).

In vitro-culture experiments with inhibitor concentrations up to 400 μM for at least 96 h had no significant effect on the vitality, morphology, pairing stability or egg production of treated worms (data not shown). Only 500 μM or higher concentrations of 3289-8625 led to obvious alterations of worm morphology and survival within 24 h (**Figure 4**). Compared to control couples treated worms separated during this period, and male worms were no longer attached to the Petri dish. Using confocal laser scanning microscopy (CLSM) more detailed analyses revealed drastic influences of the drug on the morphology of the reproductive organs of both genders. The testes of treated males contained large, pore-like structures whereas in the ovaries of females the greatest effects were observed in the anterior part which contained numerous damaged cells and cell fragments (**Figure 4**).

Eggs were more susceptible to 3289-8625 treatment. Eggs laid by treated worms were cultured for 3–4 days (at 37°C and 5% CO_2) and observed by bright-field microscopy (**Figure 5**). At this time point, control eggs had reached stages II and III according to the definition provided by Jurberg et al. (2009). This is evidenced by the increased size of both the egg and the embryo, which fills 50–70% of the egg and is transparent in appearance. Macromeres are present at the poles; these will become the outer envelope of the miracidium (Jurberg et al., 2009). The number of eggs reaching this developmental stage decreased with increasing inhibitor concentration (**Figure 5**). At 100 μM only 50% of the eggs developed, whilst at concentrations of 200 μM or higher, less than 10% reached stage II. The immature eggs were smaller and appeared brownish in color, probably due to an interrupted degradation of vitelline cells.

GVBD ASSAYS CONFIRMED THE KINASE ACTIVITIES OF SmFGFR-A AND SmFGFR-B AND THEIR INHIBITION BY BIBF1120

To get first hints for the functions of FGFR-signaling in the reproduction biology of adult schistosomes, *S. mansoni* couples were treated *in vitro* with the commercially available angiokinase inhibitor BIBF1120 (Hilberg et al., 2008; Roth et al., 2009) for at least 4 days. This compound specifically blocks the enzymatic activity of human vascular endothelial growth factor (VEGF)-, platelet derived growth factor (PDGF)- and FGF-receptors with IC_{50} values between 20 and 100 nM in enzymatic assays by binding to the ATP-binding site of the kinase domains of RTKs in cell cultures (Hilberg et al., 2008; Roth et al., 2009). Using BLAST analyses no homologs of VEGFRs and PDGFRs were found in the genome of *S. mansoni*. Thus, SmFGFR-A and SmFGFR-B are likely to represent the major targets of this inhibitor in schistosomes.

To test the activity of BIBF1120, we expressed the TK domains of the two schistosome FGFR homologs in *Xenopus* oocytes

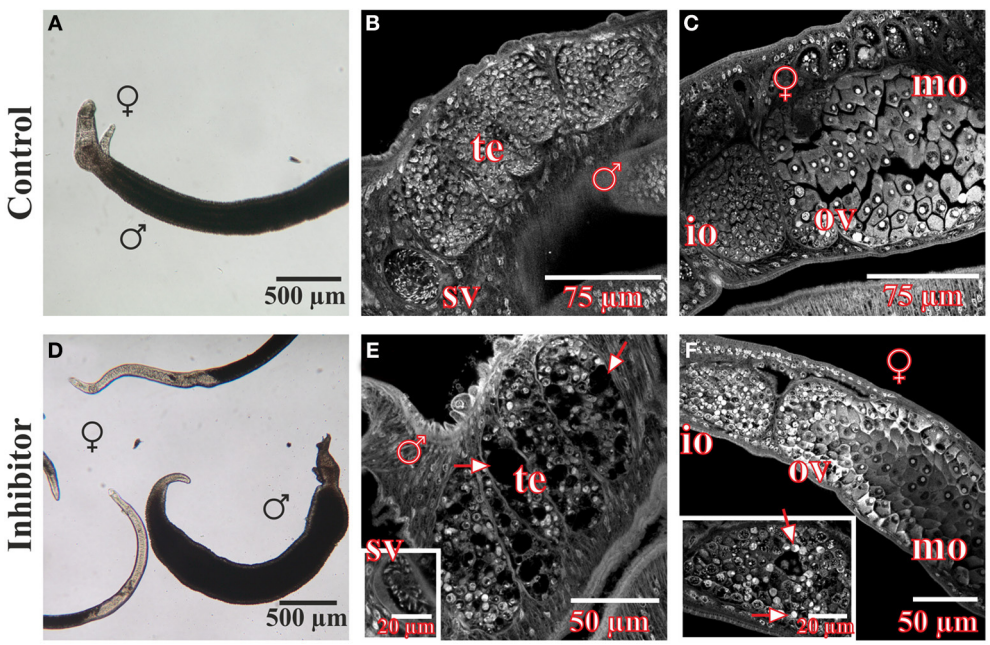

FIGURE 4 | Morphology of *S. mansoni* couples after treatment with the Frizzled-Dvl inhibitor 3289-8625 *in vitro*. *S. mansoni* couples were cultured *in vitro* for 96 h with different concentrations of the Frizzled-Dvl inhibitor 3289-8625 and subsequently studied by bright field microscopy and CLSM for morphological changes. Whereas no morphological alterations were seen in control worms **(A–C)**, inhibitor treatment (500 μM) led to a reduced vitality accompanied by a separation of couples **(D)**. Furthermore, CLSM-analyses revealed severe effects of the inhibitor on the morphology of the gonads. The testes of treated males contained large pore-like structures (**E**, arrows) whereas ovaries of females were mostly affected in the anterior part containing several damaged cells and cell fragments (**F**, arrows). [te, testes; sv, seminal vesicle; ov, ovary; io, immature oocytes; mo, mature oocytes; ♂, male; ♀, female].

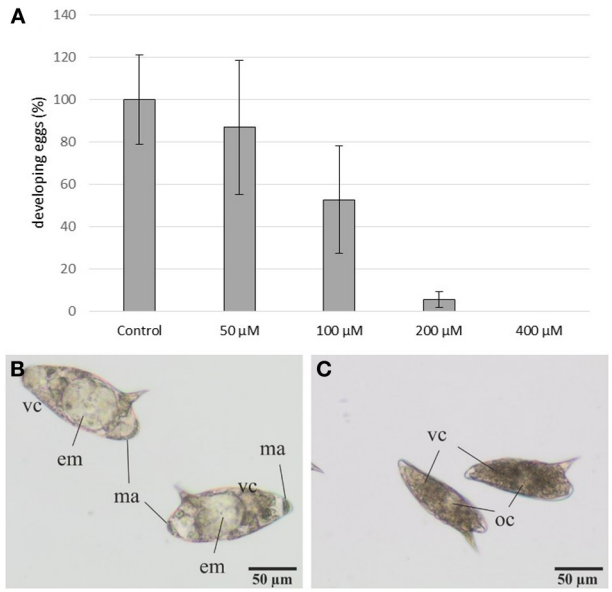

FIGURE 5 | Influence of the inhibitor 3289-8625 on the development of *S. mansoni* eggs. *S. mansoni* couples were treated with different inhibitor concentrations *in vitro*. After 24 h worms were removed from the dishes, and the deposited eggs were cultured for an additional 3-4 day period. Subsequent analyses revealed a concentration-dependent effect of 3289-8625 on egg development compared to the DMSO control **(A)**. The statistical evaluation of three independent experiments is shown (error bars indicated). Untreated eggs showed continued development and were categorized into the stages II and III of embryogenesis as defined by Jurberg et al. (2009) **(B)**. Upon increasing inhibitor concentrations embryogenesis was remarkably affected resulting in a larger proportion of undeveloped eggs **(C)**. [em: embryo; ma: macromeres; oc: oocyte; vc: vitelline cells].

and performed germinal vesicle breakdown (GVBD) assays. This system was successfully used to express schistosome TKs and to monitor their enzymatic activity under the influence of kinase inhibitors (Long et al., 2010; Beckmann et al., 2011; Vanderstraete et al., 2013). To induce GVBD in oocytes it is necessary to express an active form of the kinase. For this reason a constitutively active variant of the SmFGFR-B TK domain was generated by site-directed mutagenesis, in which the lysine of the sequence motif YYRK^{519} was changed to glutamate (YYRE^{519}). This amino acid exchange corresponds to the YYKK^{650} to YYKE^{650} mutation in the activation loop of the human (h)FGFR3, which led to a ligand-independent receptor activation (Neilson and Friesel, 1996; Webster et al., 1996) (**Figure S1**). In (h)FGFR3 phosphorylation of both regulatory tyrosine residues N-terminally of K^{650} unblock the catalytic site of the enzyme. The K^{650}E mutation mimics this phosphorylation by introducing a negative charge and induces conformational changes which allow access to the catalytic site. In the wild type form of the receptor these conformational changes occur upon ligand binding-induced receptor dimerization, followed by trans-phosphorylation of the aforementioned tyrosines. Comparable mutations have also led to constitutively active variants of other RTKs including the human insulin receptor (Hubbard et al., 1994) and the schistosome venus

Table 1 | Influence of BIBF1120 on the capacity of the TK domains of FGFR-A and FGFR-B to induce GVBD in *Xenopus* oocytes.

BIBF1120	0 µM	0.5 µM	1 µM	2 µM	5 µM	10 µM
FGFR-A_TK-wt	100	100	100	82.5	0	0
FGFR-B_TK-active	100	100	70	0	0	0

FGFR-TK induced GVBD (numbers represent % GVBD; mean of two independent experiments) in Xenopus oocytes was completely blocked by BIBF1120 at concentrations of 2 and 5 µM, respectively.

kinase receptors (VKRs) SmVKR1 and SmVKR2 (Ahier et al., 2009; Beckmann et al., 2011; Gouignard et al., 2012). As expected the expression of FGFR-B_TK-active but not that of FGFR-B_TK-wt was sufficient to induce GVBD in *Xenopus* oocytes (**Table 1**).

Interestingly, alignments of several FGFR kinase domains revealed that SmFGFR-A differs from other receptors with respect to the conserved consensus sequence YY(K/R)K of the activation loop. In contrast, FGFR-A has the motif GYME^{781} and contains a negatively charged glutamate C-terminally of the presumptive regulatory tyrosine residue. This raised the question of how the enzymatic activity of the TK domain is regulated and suggested the possibility that the native receptor is *per se* constitutively active. To obtain evidence for this hypothesis, the wild type TK domain of SmFGFR-A was expressed in the *Xenopus* system and induced GVBD in oocytes to nearly 100%. The GVBD-inducing capacity of the TK domains of SmFGFR-B_TK-active and SmFGFR-A_TK-wt was completely blocked at BIBF1120 concentrations of 2 µM and 5 µM, respectively (**Table 1**).

BIBF1120 HAD SIGNIFICANT EFFECTS ON ADULT WORMS *IN VITRO*

To study the influence of BIBF1120 on the reproduction and vitality of adult schistosomes, couples were treated with different inhibitor concentrations (1, 5, and 10 µM) *in vitro* for at least 96 h; egg production as well as pairing stability were determined daily (**Figure 6**). A drug concentration of 10 µM led to severe effects on worm morphology and survival. Within the first 24 h couples separated, and the worms were no longer attached to the bottom of the Petri dish. After 48 h treated worms showed extensive gut swelling and a curled body shape. At this time point other signs of viability such as gut peristalsis, muscular activity as well as egg production had almost completely stopped. The use of 5 µM BIBF1120 led to similar phenotypes, however, a time delay in the inhibitor effect was observed. Half of the treated couples started to separate within 48 h and 1 day later only 10% of the couples remained paired. An even more dramatic effect was observed on the reproductive capacity as egg production declined to about 20% compared to the control in the first 24 h. Alterations of the gut as well as general restriction of the viability were observed between 48 h and 72 h following treatment.

With respect to the influence of BIBF1120 on schistosome reproduction, the most interesting results were those obtained by supplying 1 µM of the inhibitor to *S. mansoni* couples. Treated couples stayed stable during the whole experiment, and worms showed no morphological alterations. Nevertheless, compared to the controls egg production was already reduced to less than 50% after 24 h treatment.

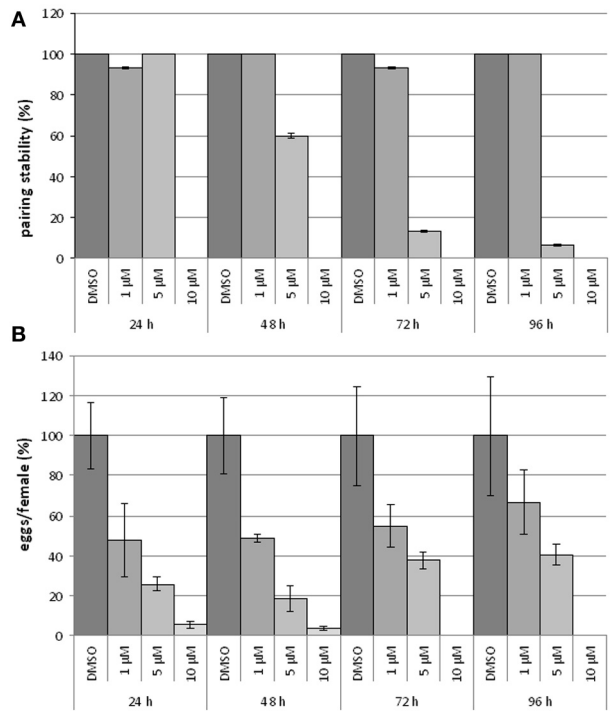

FIGURE 6 | Influence of BIBF1120 on pairing stability and egg production on *S. mansoni* couples *in vitro*. Treatment of *S. mansoni* couples with BIBF1120 for 96 h *in vitro* revealed a concentration-dependent effect of the FGFR inhibitor on pairing stability **(A)** and egg production **(B)** compared to the control (DMSO). Whereas 1 μM BIBF1120 had no influence on pairing, couples treated with 5 μM separated during the experiment period. The highest inhibitor concentration (10 μM) supplied, led to a separation of all couples within the first 24 h **(A)**. An obvious effect of BIBF1120 on egg production was also detected after 24 h. At this time point the inhibitor treatment led to a decrease of eggs down to 50% (1 μM) and to 20% (5 μM) compared to the control. At higher concentrations egg production was nearly completely disrupted **(B)**. In both diagrams the statistical evaluation of three independent experiments is shown (error bars indicated).

CLSM ANALYSES EXHIBITED SEVERE MORPHOLOGICAL EFFECTS IN BIBF1120-TREATED *S. MANSONI* COUPLES

After treatment for 96 h with the FGFR inhibitor BIBF1120, *S. mansoni* couples were analyzed in more detail using CLSM to investigate the influence of the compound on worm morphology (**Figure 7**). Compared to the DMSO control, gonad morphology was influenced following treatment with 1 μM BIBF1120, a concentration at which no inhibitor-phenotypes were observed by light microscopy. Testicular lobes of males were smaller in diameter, and seminal vesicles rarely contained mature sperms. The anterior part of ovaries in treated females contained a mass of degenerated oocytes which were not clearly distinguishable from each other.

As already observed by bright-field microscopy, an inhibitor concentration of 5 μM exerted severe effects on worm morphology and viability. Separated males and females had dramatically swollen guts. Cells of the gonads were also severely affected and appeared damaged.

EdU-INCORPORATION OF BIBF1120-TREATED *S. MANSONI* COUPLES

In a previous study, FGFR-signaling has been linked to somatic stem cell control in adult schistosomes (Collins et al., 2013). To investigate whether FGFR-signaling also plays a role in reproductive organs, *S. mansoni* couples were cultured with EdU-containing medium in the absence or presence of BIBF1120 aiming to investigate its potential effect on mitotically active cells with stem-cell characteristics (**Figure 8**). A large number of EdU-labeled cells were detected in males and females of the control group. Signals were observed in the vitellarium and the anterior part of the ovary, which contain the stem cell-like S1-vitellocytes and the oogonia, respectively. This indicated high mitotic activity in these organs, which was expected since this is a prerequisite for paired, sexually mature females to produce high amounts of S4-vitellocytes and primary oocytes for egg production (Erasmus, 1973; Popiel and Basch, 1984; Kunz, 2001; Knobloch et al., 2002, 2007).

In males high amounts of mitotically active cells were identified in the testes (**Figure 8**), which certainly represent spermatogonia. Furthermore, EdU^+-cells were detected within the parenchyma of both genders. In contrast inhibitor-treated worms showed a drastic decline of EdU^+-cells in those tissues except of the ovary, which seemed to be less affected than other organs.

DISCUSSION

In the past, several studies have focused on the identification and characterization of biological processes involved in the male-induced maturation of the female, as this is a prerequisite for egg production. These efforts included global transcriptomic approaches providing broad insights into pairing-regulated gene expression in male and female *S. mansoni* (Fitzpatrick and Hoffmann, 2006; Waisberg et al., 2007; Williams et al., 2007; Leutner et al., 2013). Nevertheless, due to the restricted access to inner organs and structures, it has not been possible so far to study the influence of pairing on tissue-specific gene expression, especially in the reproductive organs. To overcome this restriction we recently established a protocol for the isolation of testes and ovaries from adult schistosomes (Hahnel et al., 2013).

Based on this approach, in the present study comparative qRT-PCR analyses were performed to investigate the influence of pairing on the gonad-specific expression of selected genes, which are presumably involved in developmental and differentiation processes. These included genes coding for different types of transmembrane proteins like a Notch receptor homolog, the frizzled receptor SmFz1, two FGFRs (SmFGFR-A and SmFGFR-B), as well as the membrane progestin receptor component SmPMRC1. Additionally, a Musashi homolog was included in the analyses. As a RNA-binding protein, in *Drosophila* Musashi controls among other functions, translational events in the germ line (Gunter and McLaughlin, 2011).

Determining the relative transcription levels of these genes in whole worms revealed their expression in adult *S. mansoni* of both genders independently of the pairing status. Additionally, SmFGFR-A, SmFGFR-B, SmPMRC1, and Musashi were up-regulated in males and females by pairing providing evidence for their roles in biological processes related to pairing and sexual maturation.

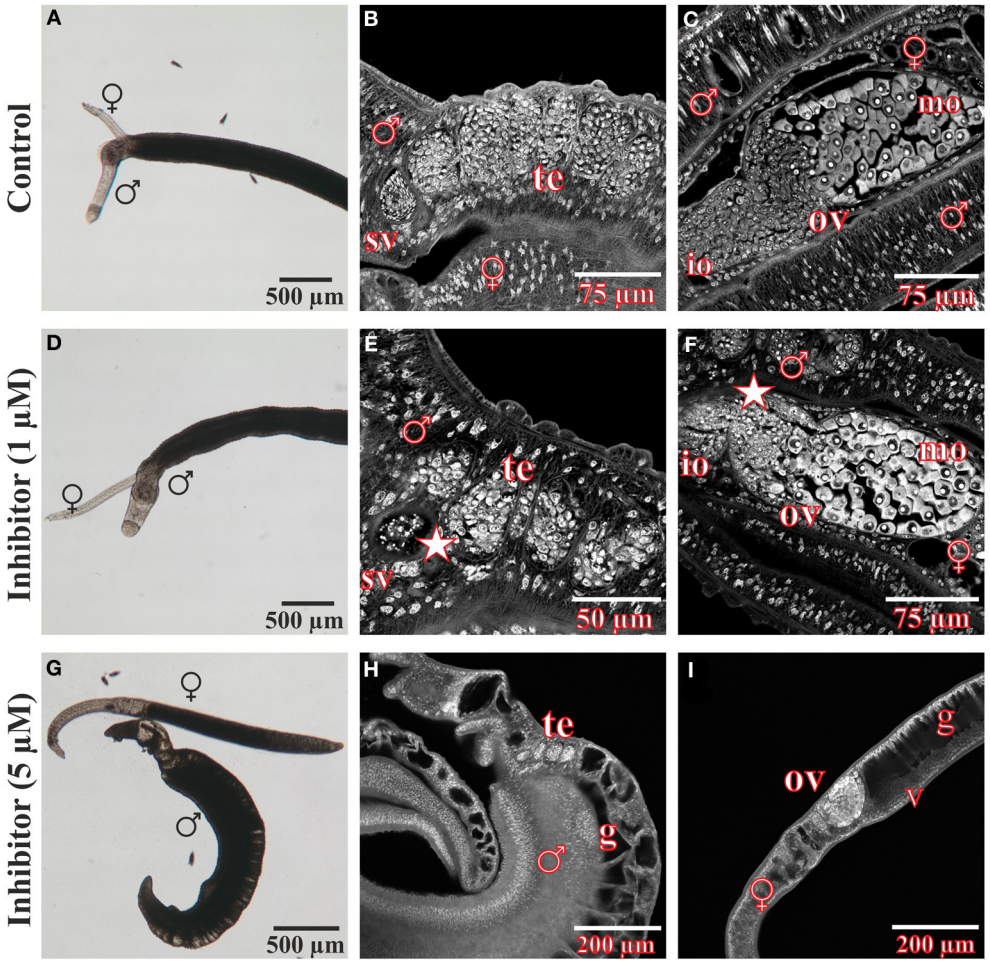

FIGURE 7 | Morphological analyses of *S. mansoni* couples after *in vitro* treatment with the inhibitor BIBF1120. Couples of *S. mansoni* were treated with different concentrations of the FGFR inhibitor BIBF1120 for 96 h *in vitro* **(D–I)** und subsequently analyzed for morphological alterations. Worms supplied with 1 µM inhibitor showed no obvious changes in vitality and morphology compared to those of the DMSO control using bright field microscopy **(A,D)**. A more detailed examination by CLSM revealed an effect of BIBF1120 treatment on the gonads of both genders. Testicular lobes of treated males were reduced in their diameter and the seminal vesicle contained fewer mature sperms (**E**, asterix). In females 1 µM of inhibitor led to a partial degradation of immature oocytes in the anterior part of the ovary (**F**, asterix) (DMSO control: **B,C**). In contrast 5 µM BIBF11120 had severe effects on worm vitality and pairing stability as well as morphology in general **(G–I)**. Those worms showed a dramatically swollen intestinal tract and cells of the gonads were severely damaged. [te, testes; sv, seminal vesicle; ov, ovary; io, immature oocytes; mo, mature oocytes v, vitellarium; ga, gastrodermis; ♂, male; ♀, female].

In addition, at the level of the gonads all investigated genes were found to be transcribed in testes and ovaries independently of the pairing status. This indicated their involvement in biological functions in these reproductive organs. Comparing transcript amounts in testes of um and em, we discovered that all genes analyzed were up-regulated upon pairing. For em this may be explained by an increased spermatogenesis. In a former morphological study it was described that testicular lobes of em contain more cells than those of um (Neves et al., 2005). In ef gonads some genes like SmPMRC1 and Musashi were highly up-regulated in mature ovaries. This may be due to the development of primary oocytes in these organs that are formed upon pairing. Previous studies have shown that steroid hormone signaling and translational regulation by Musashi are linked to oocyte maturation in model organisms (Hammes, 2004; Charlesworth et al., 2006; Arumugam et al., 2010; Parthasarathy et al., 2010). Other genes like Notch or both FGFRs were less affected by pairing, possibly because they are linked to stem cell control and stem cell fate (Dvorak et al., 2006; Gotoh, 2009; Liu et al., 2010; Waters and Reinke, 2011; Dalton, 2013; Koch et al., 2013).

The most remarkable results with respect to pairing-induced gene expression in gonads were those obtained for SmFz1 and Notch, emphasizing the advantages of tissue-specific analyses. Both genes seemed to be unaffected in their transcription by pairing when transcription rates of whole worms were compared. In contrast, transcription of both genes in testes and ovaries seemed to be strongly influenced by maturation processes. This demonstrates that "zooming-in" into specific tissues can unmask aspects of pairing-regulated gene expression, which otherwise remain undiscovered. Therefore, organ isolation combined

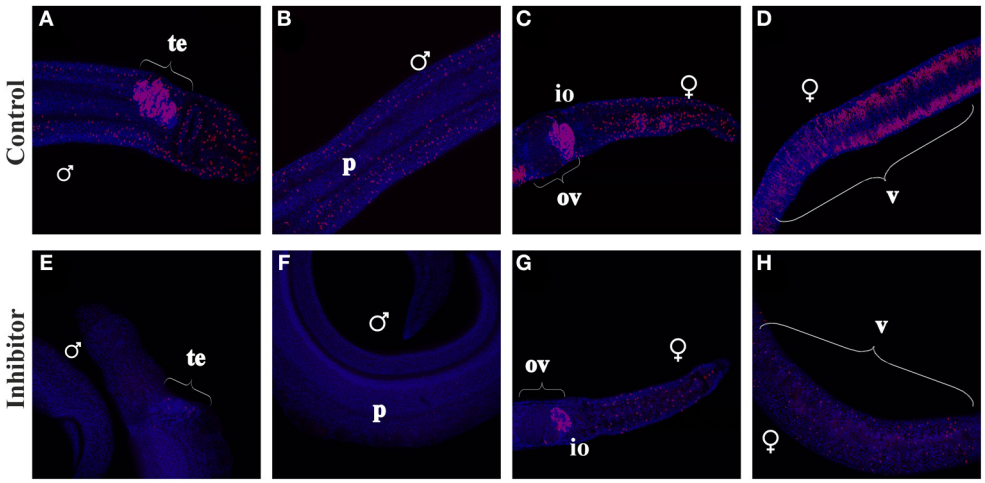

FIGURE 8 | EdU-incorporation of BIBF1120-treated *S. mansoni* couples. *S. mansoni* couples were co-cultured *in vitro* for 48 h with BIBF1120, with the addition of EdU after 24 h, to investigate the influence of the inhibitor on mitotically active cells. In worms of the control group EdU^+-cells were detected in the parenchyma and the gonads of both genders as well as in the vitellarium of the female **(A–D)**. Application of 5 μM BIBF1120 led to a drastic decline of EdU^+-cells in all tissues, although the effect on the ovary was the weakest **(E–H)**. [te, testes; ov, ovary; io, immature oocytes; v, vitellarium; p, parenchyma; ♂, male; ♀, female].

with subsequent gene expression analyses lead to a more reliable identification of pairing-dependently expressed genes involved in processes linked to sexual reproduction. Since the latter is the prerequisite for initiating and maintaining egg production, this approach has the capacity to enlarge the repertoire of candidate genes envisaged for further evaluation with respect to new antischistosomals.

Because of its pairing-dependent expression in the gonads, SmFz1 was one of three genes characterized further. Sequence analyses confirmed homology to members of the frizzled family of seven transmembrane receptors for Wnt lipoglycoprotein-type growth factors (Schulte, 2010). Wnt/frizzled signaling is highly conserved throughout the animal kingdom and involved in a broad range of developmental processes during embryogenesis, organogenesis (Huang and Klein, 2004; Almuedo-Castillo et al., 2012) but also in carcinogenesis (Klaus and Birchmeier, 2008).

In *S. mansoni* at least four different frizzled receptors, SmFz1 - SmFz4, were identified by previous *in silico* analyses (Zamanian et al., 2011; Hahnel et al., unpublished), but up to now none of those genes have been investigated further. In the present study *in situ*-hybridization experiments localized SmFz1 transcripts in testes and ovaries of adult *S. mansoni* and confirmed the results of the gonad-specific qRT-PCRs. Because transcription was not detected in other tissues, we conclude that SmFz1 is gonad-specifically expressed. Since no transcripts of other frizzled receptors could be localized in the gonads by organ-specific RT-PCRs (data not shown), it seems likely that Fz-signaling in the gonads of *S. mansoni* is exclusively mediated by SmFz1.

Although frizzled receptors belong to the superfamily of GPCRs, typical canonical Frizzled-signaling pathways occur independently of heterotrimeric G proteins, but rather by interacting with the cytosolic adapter protein Disheveled (Dvl) (Gao and Chen, 2010). Since a characteristic Dvl-binding motif was found within the C-terminus of SmFz1, and SmDvl1 as well as SmDvl2 transcripts were identified in testes and ovaries by organ-specific RT-PCRs (data not shown), evidence exists that at least one SmFz1-dependent canonical frizzled pathway is expressed in the gonads of adult *S. mansoni*. Using an inhibitor (3289-8625), which specifically blocks Frizzled-Dvl interactions, we started to investigate the role of associated signaling pathways in adult *S.* mansoni *in vitro*. Adult worms treated with this inhibitor showed dramatic morphological alterations in the gonads accompanied by a massive destruction of cells in testes and ovaries. Because Frizzled-Dvl signaling is involved in controlling fundamental processes like organogenesis and gametogenesis in the gonads of diverse organisms (Schalburg et al., 2006; Golestaneh et al., 2009; Sirotkin, 2011) the observed phenotype might be due to inhibition of SmFz1-signaling in these tissues. Furthermore, worm vitality and morphology were affected, leading to the conclusion that further Frizzled-Dvl pathways were also expressed in other tissues of adult *S. mansoni* and additionally targeted by the inhibitor. Finally, effects were also detected in eggs laid by treated couples. As 3289-8625 prevented normal embryo development in such eggs, the obtained results provide first evidence for the involvement of Frizzled-Dvl signaling in early embryogenesis of *S. mansoni* as has been reported also for other organisms (Huang and Klein, 2004; van Amerongen and Nusse, 2009; Wansleeben and Meijlink, 2011; Almuedo-Castillo et al., 2012). Although an IC_{50} of 12.5 μM for 3289-8625 in human cell culture was reported (Grandy et al., 2009), the effects observed here on worm morphology and embryogenesis occurred at relatively high inhibitor concentrations. This discrepancy might be explained by structural differences of the inhibitor target sites of human and schistosome homologs. Even though the PDZ domains of SmDvl1 and SmDvl2 shared high similarity to those of human Dvl2 with sequence identities of 72.5 and 85%, respectively, we cannot exclude that key residues are missing in the schistosome homologs which are involved in inhibitor binding. In addition, the uptake

of the compound by the parasite, its chemical stability in worm culture medium and tissue, as well as its distribution inside the worm, are potential factors influencing inhibitor activity.

As Frizzled receptors, RTKs of the FGFR family are highly conserved throughout the *Eumetazoa* (Itoh and Ornitz, 2004, 2011) and control a wide spectrum of cellular processes including cell division, differentiation, maintenance, migration and apoptosis (Powers et al., 2000). Therefore, they fulfill a crucial role in organogenesis during embryonic development of both, invertebrates and vertebrates, but are also involved in the regulation of physiological processes like tissue regeneration, homeostasis, and angiogenesis in the adult organism (Turner and Grose, 2010). Additionally, several studies have linked FGFR signaling to stem cell control in different model systems (Dvorak et al., 2006; Gotoh, 2009; Dalton, 2013). FGFRs are also expressed in planarian neoblasts. They represent pluripotent stem cells involved in somatic tissue regeneration of these free-living flatworms (Ogawa et al., 2002; Adell et al., 2010).

In schistosomes, two homologs of the FGFR-family exist, SmFGFR-A and SmFGR-B. Sequence analyses revealed that SmFGFR-A exhibits characteristic features of FGFRs including two extracellular IG-like domains sufficient for ligand binding and receptor dimerization (Eswarakumar et al., 2005). SmFGFR-B seems to represent a truncated form as the extracellular N-terminus lacks identifiable domains. However, GVBD-assays performed in this study showed that both FGFRs possessed enzymatic activity independent of their extracellular domains, which provides evidence for their biological activity. Interestingly, our data suggest that regulation of the SmFGFR-A kinase activity occurs in a non-FGFR like fashion. Normally, FGFRs share the common regulatory principle of most RTKs that depends on ligand-induced receptor dimerization followed by a trans-phosphorylation of the activation loops of both TK domains, which is sufficient for enzymatic activity (Krauss, 2008). In contrast to SmFGFR-B and other non-schistosome FGFRs, SmFGFR–A contains a constitutively active TK domain, which enabled the induction of GVBD in its wild type form. This can be explained by the unusual structure of SmFGFR–A, as the TK domain contains variations of the amino acid sequence within the activation loop. Instead of a positive charged amino acid residue found in common FGFRs, a negatively charged glutamate is present at position GYME^{781} of SmFGFR–A. Thus, charging of regulatory tyrosines within the activation loop via phosphorylation seems not to be necessary for activation.

A similar phenomenon has been reported for epidermal growth factor receptors (EGFRs), where enzymatic activity is triggered by conformational changes of both TK domains following receptor dimerization but independent of phosphorylation processes inside the activation loop (Bose and Zhang, 2009). Because SmFGFR-A shares no remarkable homology to EGFRs it remains unclear if the receptor is regulated by similar mechanisms or yet another unknown way. Although SmFGFR-A and SmFGFR-B represent members of the FGFR family, both differ from typical structures by alterations in their TK domains or their extracellular region, respectively. This leads to the conclusion that regulation of FGFR signaling in schistosomes may occur by as yet unknown mechanisms, which will be the focus of further studies.

A former study identified SmFGFR-A as a key molecule in the regulation and maintenance of proliferating somatic cells (PSCs) in adult *S. mansoni* (Collins et al., 2013). The SmFGFR–A expressing cells showed neoblast-like stem cell characteristics and may represent a source for tissue regeneration (Collins et al., 2013). They were found to be distributed throughout the parenchyma of adult males and females, and formed clusters near the intestine (Collins et al., 2013). Interestingly, we were able to detect transcripts of SmFGFR-B in the parenchyma by *in situ* hybridization, which might be a first hint that the second schistosome FGFR is also involved in PSC regulation. Regarding a potential role of FGFRs in reproduction of schistosomes, Collins et al. (2013) detected SmFGFR-A expression in the testes of male worms, which was confirmed by our experiments. Additionally, we were able to localize SmFGFR–A transcripts in the ovary of females. Furthermore, transcripts of SmFGFR-B were also detected in these tissues, which provide evidence that both receptors function in signal transduction processes in the schistosome gonads. By analogy to PSC regulation, we hypothesized that the mitotic activity of germinal stem cells, might also be regulated by FGFR signaling. Interestingly, while both FGFRs were co-localized in the gonads, our localization results indicate that only SmFGFR-B is expressed in the vitellarium, where it could be involved in the regulation of stem cell-like S1-vitellocytes (Kunz, 2001).

To investigate the role of FGFR-signaling in schistosome reproduction we performed functional analyses using the angiokinase inhibitor BIBF1120 (Hilberg et al., 2008; Roth et al., 2009). This compound (also named Vargatef) inhibits enzymatic activity of human VEGFRs, PDGFRs, and FGFRs by blocking the ATP-binding site of the TK domain. As no VEGFR and PDGFR homologs are encoded in the *S. mansoni* genome we assume that SmFGFR-A and SmFGFR-B may represent major targets of the inhibitor in this parasite. GVBD-assays confirmed that BIBF1120 was able to block enzymatic activity of both FGFRs in a concentration-dependent manner. Interestingly, SmFGFR-B was affected at lower concentrations, showing a higher sensitivity of this homolog to the inhibitor. According to our localization studies, the GVBD-data, and the known association of FGFR-A to PSCs, we hypothesize that BIBF1120 possesses the capacity to alter PSCs function in adult *S. mansoni* but also FGFR-expressing cells in the reproductive organs. Treatment of *S. mansoni* couples with BIBF1120 *in vitro* showed severe effects on worm vitality, morphology, and reproduction using concentrations of 5–10 μM. Most obviously, treated worms exhibited a drastically swollen intestinal tract. This may explain the decreased viability observed following treatment. Because FGFR-B expression was also detected in the gastrodermis it seems to be conclusive that the inhibitor had a direct effect on this tissue. Furthermore, it seems likely that these alterations were triggered by a targeting of FGFR-signaling in PSCs, since it has been suggested by Collins et al. (2013) that gastrodermis renewal depends on PSC activity. Besides this, inhibitor treatment also affected the gonads, which contained damaged cells. This confirms that FGFR-signaling plays an important role in these tissues as well. Although FGFR-B expression was localized in the vitellarium, CLSM analysis of treated worms (5–10 μM) showed no obvious morphological changes in this organ. Lower inhibitor concentrations (1 μM)

led to less dramatic phenotypes on vitality and worm morphology. Nevertheless, egg production of treated couples was strongly affected. In males, testes morphology and sperm production were altered upon treatment. The anterior part of ovaries in treated females contained degenerated immature oocytes, not clearly distinguishable from each other. Both phenotypes could be explained by an effect of BIBF1120 on mitotically active cells in these organs.

To test the hypothesis that BIBF1120 affects germinal stem cells we co-cultured inhibitor-treated couples with EdU. In correspondence to the results obtained by Collins et al. (2013) we detected EdU^{+}-cells widely distributed throughout the parenchyma, along the intestine as well as in the gonads and the vitellarium of adult *S. mansoni*. Targeting FGFR-signaling using BIBF1120, however, led to a remarkable decline of PSCs in the parenchyma of both genders. Beyond this we observed the disappearance of mitotically active cells in the testes and in the vitellarium, spermatogonia and S1-vitellocytes, respectively. In contrast to these findings, oogonia in the ovaries of mature females seemed to be less sensitive to inhibitor treatment. Nevertheless, the effects of BIBF1120 on gonadal cells and S1-vitellocytes provide an explanation for the inhibitor-induced decline in egg production.

Taking into account the limitations of inhibitor usage for gene characterization, the results obtained here provide first hints for an involvement of FGFR-signaling in the reproductive biology of adult *S. mansoni*. Furthermore, the data of the previous and the present studies suggest that FGFR-signaling should be assessed as a target for alternative strategies fighting schistosomiasis.

ACKNOWLEDGMENTS

The authors thank Christina Scheld, Bianca Kulik, and Mathias Riedl for their excellent technical assistance. This work was financially supported by a grant of the Deutsche Forschungsgemeinschaft (GR-1549/7-2). Sophia Parker-Manuel receives a fellowship from the Alexander-von-Humboldt (AvH) foundation. Zhigang Lu is a member of the International Giessen Graduate Centre for The Life Sciences (GGL) and receives a fellowship from the China Scholarship Council (CSC).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fgene.2014.00170/abstract

Figure S1 | Sequence alignment of the activation loop of diverse FGFRs
Sequence alignment of the activation loop of the human FGFR 3 (hFGFR3) (AC: AAI53825.1), DjFGFR1 (AC: Q8MY86.1), and DjFGFR2 (AC: Q8MY85.1) from the planarian *Dugesia japonica* as well as SmFGFR-A and SmFGFR-B. The DFG motif (blue box) is part of the Mg^{2+} binding pocket of the FGFR TK domain and is conserved in all analyzed receptors. This amino acid sequence was changed to DNA by site-directed mutagenesis to create inactive kinase domains of SmFGFR-A and SmFGFR-B, serving as negative controls in the GVBD assays. The green box highlights the conserved regulatory motif $YYKK^{650}$ of (h)FGFR3 and the corresponding motif YYRK, occurring in both planarian receptors and SmFGFR-B. In (h)FGFR3 phosphorylation of both of the regulatory tyrosine residues N-terminal of K^{650} unblocks the catalytic site of the enzyme. The $K^{650}E$ mutation mimics this phosphorylation by introducing a negative charge and leads to a constitutively active kinase. For this reason a constitutively active variant of the SmFGFR-B TK domain (SmFGFR-B_TK-active) was generated by changing $YYRK^{519}$ to $YYRE^{519}$. In contrast, SmFGFR-A has the motif $GYME^{781}$, containing a negatively charged glutamate C-terminal to the presumptive regulatory tyrosine residue (underlined). Thus, SmFGFR-A possesses a constitutively active TK domain in its wild type form, which is sufficient to induce GVBD.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 April 2014; accepted: 21 May 2014; published online: 10 June 2014.
*Citation: Hahnel S, Quack T, Parker-Manuel SJ, Lu Z, Vanderstraete M, Morel M, Dissous C, Cailliau K and Grevelding CG (2014) Gonad RNA-specific qRT-PCR analyses identify genes with potential functions in schistosome reproduction such as SmFz1 and SmFGFRs. Front. Genet. **5**:170. doi: 10.3389/fgene.2014.00170*
This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Genetics.

frontiers in GENETICS

OPINION ARTICLE
published: 27 May 2014
doi: 10.3389/fgene.2014.00120

Heterologous expression in *Caenorhabditis elegans* as an alternative approach to functional studies in *Schistosoma mansoni*

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Keywords: ***Schistosoma mansoni*, heterologous expression, *Caenorhabditis elegans***

INTRODUCTION

The use of heterologous expression systems and expression vectors provide a powerful tool for studying the cellular functions of specific genes in their natural cellular environment or in specialized host organisms (Porro et al., 2005). In this context, the gene of interest is cloned in combination with a suitable promoter in a heterologous host (Gräslund et al., 2008). This approach has special relevance in the study of parasitic diseases, such as Schistosomiasis, in which the functional characterization of the parasite is hampered specially because these organisms are not amenable to genetic manipulation and their entire life cycle cannot be cultured *in vitro* (Alrefaei et al., 2011; Suttiprapa et al., 2012; Liang et al., 2013). Although genetic manipulation it is difficult to be achieved in parasitic helminthes, significant advances have been reported towards development of transgenic schistosomes as gene silencing by RNA interference (RNAi) and transient and stable transfection including transgenesis mediated by genome integration using competent vectors or retrovirus (Beckmann and Grevelding, 2012; Mann et al., 2014). Therefore, protein expression in a heterologous host may help the understanding of physiological processes and identifying potential targets with biomedical and biotechnological applications.

Schistosoma mansoni is one of the causative agents of human intestinal schistosomiasis. The availability of the genome sequence and a significant amount of transcriptomic and proteomic information allowed the application of a variety of methodologies for the identification and characterization of molecules involved in different physiological mechanisms such as cell signaling essential for the *S. mansoni*'s parasite biology (Knudsen et al., 2005; Curwen et al., 2006; Cass et al., 2007; Guillou et al., 2007).

Protein kinases (PK) play key roles in signaling pathways and have been proposed as potential targets for the development of new anti-schistosome drugs (Dissous et al., 2007). Approximately 1.9% (252 proteins) of the predicted *S. mansoni* proteome corresponds to PKs. However, less than 15% of the kinases have experimental functional evidence, including the JNK subfamily member (Andrade et al., 2011). In contrast to the number of homologs in other species, Andrade and colleagues (2011) have identified only one protein belonging to the JNK subfamily (Smp_172240) in the *S. mansoni* proteome. Knockdown of SmJNK by RNA interference (RNAi) in schistosomula significantly reduced the total numbers of eggs and adult parasites in infected mice (Andrade, 2012). In addition, worms recovered from infected mice showed underdeveloped tegument and reproductive organs (Andrade, 2012). These results indicate that SmJNK might play a role in parasite transformation and survival in the mammal host. However, this line of approach has one methodological limitation. In schistosomes it is not yet possible to conduct functional complementation experiments. In order to advance our knowledge concerning SmJNK gene function we explored the nematode *Caenorhabditis elegans* as a heterologous platform to investigate whether *S. mansoni* JNK represents a physiologically functional protein.

C. elegans is a transparent, free-living, soil nematode with 1 mm in length that has emerged as an important animal model in various fields including neurobiology, developmental biology, and genetics. *C. elegans* was the first genome of a multicellular organism to be fully sequenced (The C. *elegans* Sequencing Consortium, 1998). This model offers several advantages, including well-established techniques for genetic and experimental manipulation. Transformation of *C. elegans* has been used to investigate the function of genes from a range of parasitic nematode species, including *tub-1* and *cpl-1* from *Haemonchus contortus*, *gst-3* from *Onchocerca volvulus* and *fktf-1b* from *Strongyloids stercoralis* (Grant, 1992; Kwa et al., 1995; Britton et al., 1999; Redmond

et al., 2001; Britton and Murray, 2002; Kampkötter et al., 2003; Massey et al., 2006).

The *C. elegans* genome encodes five proteins classified as belonging to the subfamily JNK (*jnk-1*, ZC416.4, T07A9.3, Y51B9A.9, and C49C3.10). Based on evolutionary relationships of the JNK protein, *C. elegans jnk-1* was found to be the orthologue of SmJNK (*data not shown*). In *C. elegans*, *jnk-1* is involved in the modulation of coordinated locomotion (Villanueva et al., 2001). *C. elegans jnk-1* deletion mutants are short-lived, more susceptible to heavy metal and heat stress (Villanueva et al., 2001; Wolf et al., 2008). Overexpression of *jnk-1* increases the resistance to oxidative stress and prolongs the worm's lifespan (Villanueva et al., 2001; Oh et al., 2005). Based on this information, we tested whether the overexpression of SmJNK in *C. elegans* would also result in similar phenotypes, enabling the demonstration that the schistosome enzyme is active and capable of complementing the function of the original gene.

STRAIN CONSTRUCTION

The expression vector must contain all the DNA sequences necessary for its own expression. The *C. elegans* transcription machinery should be able to recognize and correctly interpret the signals present in these sequences. In order to obtain specific transgenic lineages overexpressing SmJNK, we first constructed the expression cassettes containing the cDNA of *S. mansoni* SmJNK (*Sm_JNK*) downstream of the *C. elegans jnk-1* gene promoter (*Ce_jnk-1p*).

We have chosen to use the *C. elegans* promoter because we had no knowledge if the *C. elegans* transcription machinery would recognize the *S. mansoni* promoter. Additionally, it is possible that gene expression patterns may be differente in two species and the use of the *S. mansoni* promoter may produce a different phenotype due to expression in different cell types or at different levels (Cook et al., 2006). The systematic study of schistosome gene promoters is an area not much explored, but of central relevance for transgenese studies in the field. In *C. elegans*, the majority of protein coding genes are within gene-dense regions of the genome, with cis-acting regulatory regions usually close to the coding region. Consequently, the minimal promoter region required for proper expression of most RNA Polymerase II transcripts lies within a couple of kilobases upstream of the start codon (Okkema and Krause, 2005). For this reason, we selected the 3 Kb region upstream of *Ce_jnk-1* gene as a promoter region. We used *S. mansoni* cDNA to amplify the coding regions to avoid any possibility of incorrect splicing, once its intronic regions could not be recognized by the *C. elegans* splicing machinery. As a positive control, we also construct another transgenic line containing *jnk-1* cDNA of *C. elegans* (*Ce_JNK-1)* under control of the same promoter *Ce_jnk-1p*. The 3′-UTR region was not included due to the lack of exclusive restriction sites in this region.

The DNA final constructs were delivered to *C. elegans* N2 through intragonadal microinjections (Mello et al., 1991). As previous described by Oh et al. (2005) (**Figure 1A**), the plasmids were injected at 50 ng/μL into the gonad of young adult N2 worms to generate stable extrachromosomal transgenic lines. Plasmid pRF4 [*rol-6*(*su1066*) plasmid], which has a dominant mutation in *rol-6*, was coinjected at 100 ng/μL for selection by the induction of a dominant "roller" phenotype in the transgenic lineages (**Figure 1A**). We generated three independent extrachromosomal lineages for *Ce_JNK-1* (N2 *Ex01[Ce_jnk-1p::Ce_JNK-1]*, N2 *Ex02[Ce_jnk-1p::Ce_JNK-1]* and N2 *Ex03[Ce_jnk-1p::Ce_JNK-1]*) and two independent extrachromosomal lineages expressing *Sm_JNK* (N2 *Ex04[Ce_jnk-1p::Sm_JNK]* and N2 *Ex05[Ce_jnk-1p::Sm_JNK]*). We used wild type worms microinjected only with the plasmid pRF4 as negative control. All strains were maintained on nematode growth medium (NGM) plates at 20°C and fed with bacteria of the *E. coli* OP50 strain, as described by Brenner (1974).

EXPRESSION LEVELS AND LIFESPAN ANALYSIS

Expression of the *Ce_JNK-1* and *Sm_JNK* was detected in all transgenic lines (**Figures 1B,C**). However, expression levels of the transgene varied substantially among. In the transgenic lines obtained in our study, the expression level of *Ce_JNK-1* ranged from 30 times in the strain *Ce_JNK-1 Ex01* to 1.5 times in the strain N2 *Ce_JNK-1 Ex03* when compared with the control lineage (**Figure 1B**). The expression level of *Sm_JNK* also varied ranging from 40 times in the strain *Sm_JNK Ex04* to 11 times in the strain *Sm_JNK Ex04* in comparison to the control line (**Figure 1C**). Any regulatory factor present in *cis* introns were absent in our constructions, once constructions were synthesized from cDNA. It is possible that the addition of such region could further increase the level of expression observed.

Next, we evaluated whether the *Sm_JNK* overexpression could increase the *C. elegans* lifespan (**Figure 1D**). To monitor longevity under normal conditions, *Ce_JNK-1* and *Sm_JNK* animals were grown at 20°C and scored every two days. Despite the increased expression of *Ce_JNK-1* in transgenic lineages, we did not observe any increase in their longevity as earlier described by Oh et al. (2005). We also did not observe any phenotypic changes in transgenic lines overexpressing *Sm_JNK*. These results could be explained by the absence of introns or 3′-UTR region in the constructs used in this work. As previously described in the literature, the success of phenotype rescue experiments depends on factors such as the presence of regulatory elements in promoter region, the correct processing of *cis*-, and possibly *trans*-splicing, as well as 3′ formation of the pre-RNA to produce mature mRNA (Gilleard, 2004). Moreover, the steady-state levels of proteins in eukaryotic cells are also strongly dependent on translational regulatory mechanisms. The overall rate of protein synthesis as well as the translational efficiencies of individual mRNAs are regulated in response to different signals. Therefore, the over expression observed at mRNA level does not may necessarily result in increased protein levels in the correct active form. It is also worth mentioning that the microinjection technique used to obtain transgenic lines carrying repetitive extrachromosomal arrays is relatively fast and efficient. However, one of its drawbacks is that it is difficult to predict and control the level of expression among different arrays resulting in strains with distinct levels of transgene expression. Furthermore, the DNA is injected in the target tissue in an

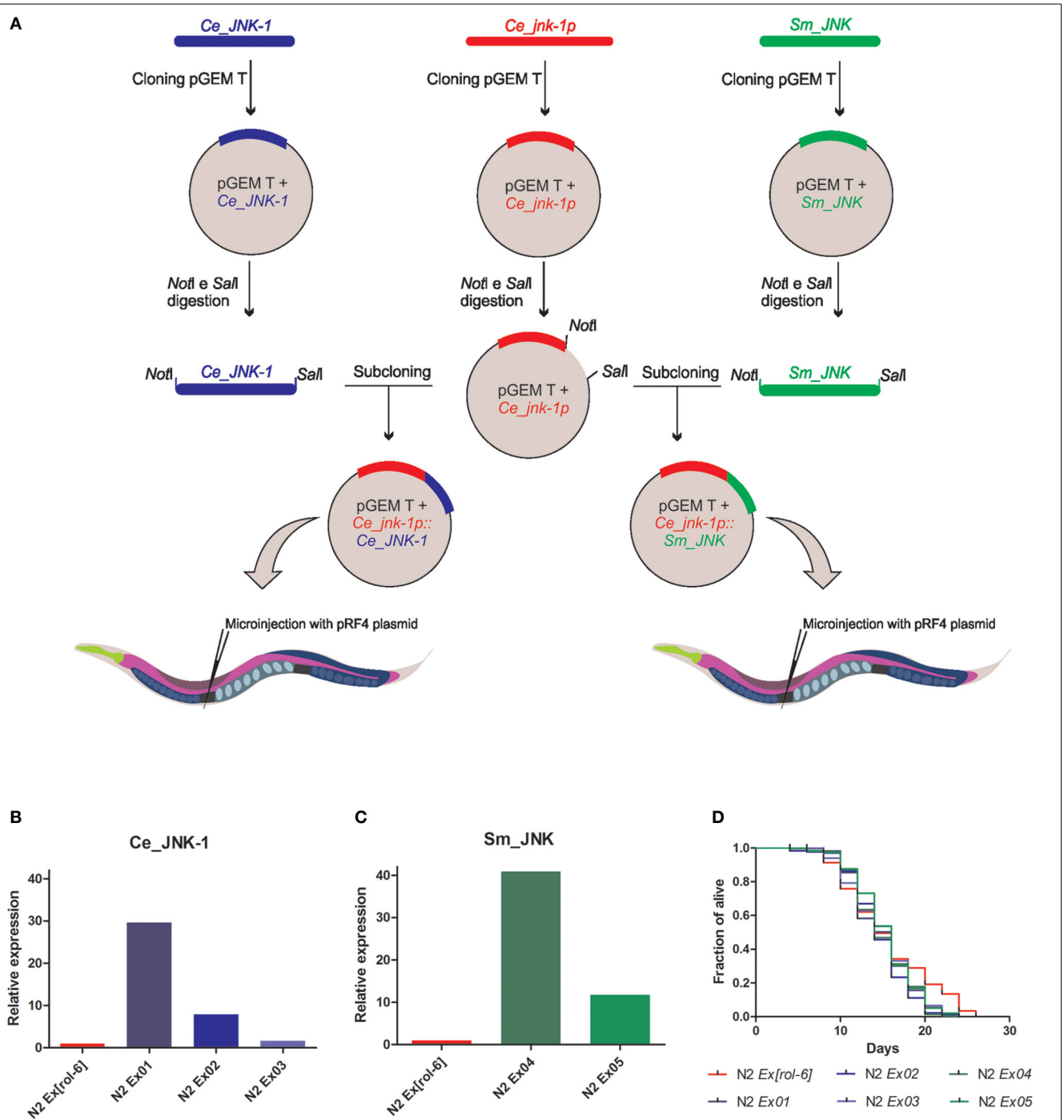

FIGURE 1 | Experimental cloning strategy and analysis of the expression level and the longevity in *C. elegans* overexpression *Sm_JNK*. (A) The promoter region of *Ce_jnk-1p* gene was amplified from genomic DNA of adult *C. elegans* N2 Bristol by PCR (5′-GCGCGCAAACTTCCATCTCCTG TTTCTC, 3′-GCGCGCGTGCACAGGATCACACACTTTA). Total RNA was extracted from schistosomula and *C. elegans* adult worms using TRIzol® reagent (Invitrogen) following the standard manufacturer's protocol. CDS of *Sm_JNK* (5′-**GCGGCCGC**ATGGCAAACAACATTCCTCC, 3′- **GTCGAC** TTAATTTTGAATATTACGTA) and *Ce_JNK-1* (5′-**GCGGCCGC**ATGGAGGAACG ATTATCCAC, 3′**GTCGAC**TCAGGAATAAATGTCATGGG) were amplified from synthesized cDNA by PCR. Fragments were subsequently cloned in pGEM®-T vector. The construction obtained was digested with restriction enzymes (*Not*I and *Sal*I) to linearize the vector containing the promoter region and to recover the CDS. Subsequently, subcloning was performed by ligation of *C. elegans* and *S. mansoni* CDS with the construct containing the promoter region. The plasmids were injected at 50 ng/μL into the gonad of young adult N2 worms to generate stable extrachromosomal transgenic lines. mRNA level of *Ce_JNK-1* **(B)** *Sm_JNK* **(C)** in wild-type animals. mRNA levels were measured in the transgenic lineages obtained for *Ce_JNK-1* (N2 *Ex01[Ce_jnk-1p::Ce_JNK-1]*, N2 *Ex02[Ce_jnk-1p::Ce_JNK-1]*, and N2 *Ex03[Ce_jnk-1p::Ce_JNK-1]*) and lineages obtained for *Sm_JNK* (N2 *Ex04[Ce_jnk-1p::Sm_JNK]* and N2 *Ex05[Ce_jnk-1p::Sm_JNK]*). Total RNA of each transgenic lines or N2 worms was isolated from approximately

(Continued)

FIGURE 1 | Continued
50 animals using TRIzol® reagent (Invitrogen). cDNA was synthesized using SuperScript™ II Reverse Transcriptase (Invitrogen). RT-qPCR was performed in triplicate with a ABI 7500 RT-PCR system (Applied Biosystems) using SYBR® Green (Applied Biosystems) and the data was analyzed using the comparative Ct method (Livak and Schmittgen, 2001). Relative mRNA levels were normalized to *cdc-42* mRNA levels.
(D) Worms in L4 stage or young adults were then transferred to new NGM plates containing 0.1 mg/mL 5′-flurodeoxyuridine (FUDR) to prevent progeny growth (Hosono et al., 1982). Animals were tapped every two days and scored as dead when they did not respond to the platinum wire pick. We determined worm's survival from the point when they were transferred to the FUDR plate and lifespan was defined as the account of days that the worms survived starting at day 1. The lifespan assays were repeated three times and statics analysis were done using the Log-rank (Mantel-Cox) test.

established concentration, but it is not possible to control the amount of DNA successfully injected into each gonad, nor the amount incorporated into arrays in each strain obtained (Evans, 2006).

CONCLUSIONS

Heterologous expression experiments have been performed an alternative approach to characterize schistosome genes. In the present paper, we described for the first time the use of *C. elegans* as an alternative heterologous host to functional studies in *S. mansoni*. The nematode *C. elegans* is more closely related to *S. mansoni* than bacteria, yeast, protozoa, or mammal cells, some of which have been used in heterologous species experiments. The excellent assembly and annotation of the *C. elegans* genome sequence is a valuable resource for studying the developmental and functional biology of parasites.

In addition to the technical difficulties, there are problems regarding to the functional extrapolation of a parasite gene expressed in transgenic *C. elegans*. As all heterologous expression system, one must be careful when extrapolating data, particularly in the functional analysis of distantly related species, which genes conserved in sequence level may be involved in different biological activities (Britton and Murray, 2002). Thus, the ability of a gene from another species to rescue a phenotype in a *C. elegans* mutant does not necessarily imply in a relationship of orthology or does it mean that these genes function in the same way and in similar pathways in both species. Likewise, the failure to recover a phenotype does not necessarily suggest that the genes are involved in different processes. Since there are mechanisms for co-evolution between molecules, genes with similar functions in similar pathways cannot perform its function, because it does not interact with their downstream targets in *C. elegans* (Gilleard, 2004). Nevertheless, the use of *C. elegans* still provides a conceptual and practical framework for functional studies of parasite genes.

An alternative approach to improve the success of *S. mansoni* heterologous expression experiments using *C. elegans* as host would be to perform microinjection using constructs containing: (i) coding regions cloned into vectors specific for expression in *C. elegans*; (ii) synthetically constructed DNA containing the promoter region, the coding region, and 3′-UTR region of the gene of interest.

We hope that the results obtained in this study will contribute to the designing of future experiments that intend to use heterologous expression as an alternative approach to functional studies in *S. mansoni*.

ACKNOWLEDGMENTS

The authors thank Dr. Luciano Andrade Moreira (CPqRR) for providing the microinjector and the dependencies of the Malaria laboratory and Dr. Marina de Moraes Mourão (CPqRR) and Dr. Rosiane A. da Silva Pereira (CPqRR) for valuable assistance and helpful scientific discussions. The authors are also grateful to Francislon Silva (CPqRR) for bioinformatics technical support and to Mariana de Oliveira (CPqRR) for help with illustrations. This work received support from the CNPq, INCT-DT and CPqRR-FICOCRUZ/MG.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 12 February 2014; accepted: 18 April 2014; published online: 27 May 2014.

*Citation: Gava SG, Scholte LLS, Volpini Â, de Paula Oliveira R and Oliveira G (2014) Heterologous expression in Caenorhabditis elegans as an alternative approach to functional studies in Schistosoma mansoni. Front. Genet. **5**:120. doi: 10.3389/fgene.2014.00120*

This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Genetics.

frontiers in GENETICS

MINI REVIEW ARTICLE
published: 08 May 2014
doi: 10.3389/fgene.2014.00119

Induction of protective immune responses against schistosomiasis using functionally active cysteine peptidases

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Each year schistosomiasis afflicts up to 600 million people in 74 tropical and sub-tropical countries, predominantly in the developing world. Yet we depend on a single drug, praziquantel, for its treatment and control. There is no vaccine available but one is urgently needed especially since praziquantel-resistant parasites are likely to emerge at some time in the future. The disease is caused by several worm species of the genus *Schistosoma*. These express several classes of papain-like cysteine peptidases, cathepsins B and L, in various tissues but particularly in their gastrodermis where they employ them as digestive enzymes. We have shown that sub-cutaneous injection of recombinant and functionally active *Schistosoma mansoni* cathepsin B1 (SmCB1), or a cathepsin L from a related parasite *Fasciola hepatica* (FhCL1), elicits highly significant protection (up to 73%) against an experimental challenge worm infection in murine models of schistosomiasis. The immune modulating properties of this subcutaneous injection can boost protection levels (up to 83%) when combined with other *S. mansoni* vaccine candidates, glyceraldehyde 3-phosphate dehydrogenase (SG3PDH) and peroxiredoxin (PRX-MAP). Here, we discuss these data in the context of the parasite's biology and development, and provide putative mechanism by which the native-like cysteine peptidase induce protective immune responses.

Keywords: schistosome, cysteine peptidase, cathepsin B, Th2 immune response, papain

Schistosomiasis is caused by several helminth (worm) species of the genus *Schistosoma*, including *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum* and is endemic in 74 tropical and sub-tropical countries, most prevalently in Africa, the Middle East, South America, and South-East Asia. Diagnostic tools for the detection of infection lack sensitivity and/or specificity leading the World Health Organization (WHO) to no longer provide estimates on populations infected or at risk but rather replacing this by describing the situation as "population requiring preventive chemotherapy." A recent WHO report (World Health Organization [WHO], 2012) revealed that the total number of people needing preventive chemotherapy globally for 2010 was over 237 million, of these > 108 million were school-age children, and of which only 13% received treatment. Praziquantel is the only readily available effective drug for the treatment of the three main parasites causing human schistosomiasis, and has the advantages of low cost and self-limiting side effects. However, complete cure is seldom achieved such that for moderate (100–400 eggs per gram feces, epg) and heavy (>400 epg) infections the cure rate may not exceed 60%. Consequently, a substantial proportion of treated individuals can remain infected (probably unaware) and, therefore, are at risk of the serious sequelae of chronic schistosomiasis (Barsoum et al., 2013).

We cannot remain dependent on a single drug for the treatment and control of schistosomiasis as it is likely that in some time praziquantel-resistant parasites will emerge and, therefore, a vaccine for schistosomiasis is urgently needed. Schistosomes live intravascularly throughout their time spent in the mammalian host; the mature adult worms can reside in the mesenteric or pelvic veins for decades and are highly refractory to blood-borne immune defense elements. However, there is evidence that human populations can develop immune-mediated resistance to re-infection and, at least in animals, there is evidence that vaccine-related protective immune responses can be induced against the parasites. Schistosome larvae (cercariae) attenuated with gamma, X-ray, or ultraviolet radiation are capable of infecting their host through the skin but they do not survive to migrate beyond the lung stage; most are eliminated over a protracted time period of up to three weeks or more after infection (Harrop and Wilson, 1993). Laboratory animals immunized with radiation attenuated (RA) cercariae of *S. mansoni* and *S. haematobium* are protected against challenge infection with normal cercariae, with reductions in worm burdens compared to non-vaccinated animals varying from 30–90% depending on the host, schistosome species and strain, numbers of immunizations, and time of challenge following immunization (Dean, 1983). Its proven efficacy in primates (Eberl et al., 2001; Kariuki et al., 2004) has reinforced

the validity of the RA model, and it is still the gold standard against which the protective efficacies of recombinant antigens are compared.

Although there is no doubt that the immune response elicited by RA-vaccination mediates parasite attrition, there is no firm consensus on the phenotype of the protective immune response. Initial studies determined that reduction in challenge worm burden in RA-immunized mice correlated with polarized Th1 immune responses, characterized by the production of interferon-gamma (IFN-γ) by leukocytes in the airways of the lung (Smythies et al., 1992; Wilson et al., 1996). However, significant protection in the RA model was also found in *IL-12p40*$^{-/-}$mice (Anderson et al., 1998, 1999), and nitric oxide was shown not to be a major agent causing parasite elimination (Coulson et al., 1998). Furthermore, other studies have shown that effective immunity in the RA vaccine model involved Th2 (Mountford et al., 2001) or mixed Th1/Th2 (Hoffmann et al., 1999; Hewitson et al., 2005) immune responses. In contrast, studies on humans that exhibit resistance to infection following chemotherapy consistently indicate that Th2 immune responses correlate with protection (Ganley-Leal et al., 2006; Walter et al., 2006; McManus and Loukas, 2008; Jiz et al., 2009; Black et al., 2010a,b; Figueiredo et al., 2012; Fitzsimmons et al., 2012; Pinot de Moira et al., 2013; Wilson et al., 2014). It is possible that interpretation of these data is confounded by the role of T-cells in schistosome development. While the complete absence of $CD4^+$ T-cells significantly impairs parasite growth and reproduction (Harrison and Doenhoff, 1983; Davies et al., 2001), so too does the suppression of Th2 immune responses during the pre-patent liver phase of infection (Riner et al., 2013). Both the lung and liver represent the major sites of attrition in RA-immunized mice challenged with *S. mansoni* (Laxer and Tuazon, 1992), suggesting that an alteration to the fine balance of Th1 and Th2 immune responses that occur in these anatomical sites at different times during natural infection may be sufficient to achieve protection, rather than a polarized response one way or the other.

What is clear is that irradiated cercariae elicit the production of IgG antibodies specific to parasite proteins, which induce protection when passively transferred to mice (Abath and Werkhauser, 1996). This correlates with the age-dependent development of human immunological resistance to reinfection with *S. mansoni*, which is also associated with the presence of anti-tegument IgG antibodies (Karanja et al., 2002). In addition, it has been proposed that the resistance to re-infection following chemotherapy with praziquantel, is mediated by antibodies specific to schistosome antigens released upon worm death that are not normally encountered by the host immune response (Mutapi et al., 1998; Gomes et al., 2002). These observations prompted searches to identify those molecules recognized by antibodies taken from RA cercariae-vaccinated mice, or humans resistant to re-infection. The most prominent of these were the tegument-associated antigens; Sm23 a member of the tetraspanin family of surface molecules (Da'dara et al., 2002, 2003), the fatty acid binding protein Sm14 (Tendler and Simpson, 2008) and the apical lipid bilayer-associated glucose transporter SGTP4 (Skelly et al., 1998; Krautz-Peterson et al., 2010). Despite the induction of parasite-specific cytokines and antibodies (Da'dara et al., 2002, 2003; Mahana, 2006), immunization with these antigens failed to elicit protection above the arbitrarily chosen 40% threshold (Bergquist and Colley, 1998). Moreover, recent studies of human immune responses to these candidates did not pin-point any with a particular potential as a vaccine (Ribeiro de Jesus et al., 2000; Al-Sherbiny et al., 2003).

It is likely that antigens on the surface membrane of schistosome parasites are inaccessible to antibody binding (Keating et al., 2006; Tallima and El Ridi, 2008) which ensures that the antigens are protected behind a tight, almost impermeable, sphingomyelin-based hydrogen-bond network (Tallima and El Ridi, 2008; Migliardo et al., 2014). On the other hand, molecules released by the parasite during migration and development (termed excretory-secretory products; ESP) readily interact with specific antibodies and other effectors of the host defense system. Because the lung was demonstrated as the major site of attrition of schistosomes after immunization with RA vaccines, the early developing schistosome larvae are considered vulnerable targets of innate and adaptive immunity (Dean, 1983; Coulson, 1997). We have suggested that effective immune responses directed against these ESP harm the juvenile parasites as they pass through the narrow and convoluted capillaries of the lung (El Ridi et al., 2010). Therefore, the ESP represents a potential pool of vaccine targets. A number of ESP of cercariae, *in vitro*-cultured and *ex vivo* lung-stage schistosomula, and adult worms of *S. mansoni* (Harrop et al., 1999; Knudsen et al., 2005; Curwen et al., 2006; Hansell et al., 2008; El Ridi and Tallima, 2009), *S. japonicum* (Liu et al., 2009; Liao et al., 2011) and *S. haematobium* (Young et al., 2012) have been identified; molecules common to these preparations include actin, heat shock proteins, enolase, aldolase, glutathione *S*-transferase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase (SG3PDH), 2 *cis*-peroxyredoxin (PRX), and serine and, predominantly, cysteine peptidases.

Schistosomes express several different classes of cysteine peptidases. *S. mansoni* cathepsin B1 (SmCB1), a member of the lysosomal cysteine peptidases of the papain superfamily, was found to be expressed at high levels in the caecum and protonephridia of cercariae. Expression increases in the parasite gut soon after skin penetration and schistosomular transformation corresponding to the initiation of blood feeding (Zerda et al., 1988). The peptidase was reported to be the major hemoglobin-digesting enzyme alongside another papain-like cysteine peptidase, cathepsin L1 (SmCL1), both of which are major proteins in worm soluble extracts and ESP (Day et al., 1995; Dalton et al., 1996; Caffrey et al., 1997; Brady et al., 1999a,b, 2000; Bogitsh et al., 2001). SmCL1 efficiently degrades human hemoglobin to absorbable dipeptides and amino acids and is localized to the gastrodermis and to the tegument of adult worms. It displays 44% identity at the amino acid level with a second cathepsin L (SmCL2) which, by contrast, is not detected in cercarial extracts or gut caecum, but is predominantly localized to the reproductive system of the female parasite and to the gynecophoric canal of the male, implying involvement with the worm reproductive physiology (Dalton et al., 1996; Brady et al., 1999a,b, 2000; Stack et al., 2011). More recently, a third cathepsin L member, SmCL3, was shown to be expressed in

the worm gastrodermis and was found to hydrolyze hemoglobin and serum albumin (Dvorák et al., 2009). Furthermore, a second cathepsin B (SmCB2) was localized to the schistosome tegument and may be involved in its biogenesis (Wippersteg et al., 2005).

Cathepsin B and cathepsin L activities are readily detected by enzymatic assays in *S. mansoni* cercarial extracts and may facilitate skin penetration (Dalton et al., 1997; Kasný et al., 2007, 2009; Dvorák et al., 2008). 8–10 day old cultured larvae of *S. mansoni* also exhibited a dramatic increase in expression of these hemoglobin-degrading peptidases (Zerda et al., 1988; Dalton et al., 1995; Dalton and Brindley, 1996) and RNAi-mediated knockdown experiments showed that at least for SmCB1 the parasites cannot feed *in vitro* on hemoglobin and do not survive *in vivo* without this enzyme (Correnti et al., 2005). More recently, using microarray analysis, Gobert et al. (2010) showed that genes encoding cathepsin B and cathepsin L were greatly up-regulated in *S. mansoni* larvae cultured for 5 days (65 and 37-fold, respectively). Both SmCBs and SmCLs are highly immunogenic in infected mice and antibodies are detected in sera of *S. mansoni*-infected patients (Dalton et al., 1995; Dalton and Brindley, 1996; Planchart et al., 2007; Sulbarán et al., 2010).

The cathepsin B and L cysteine peptidases require the parasite gut-associated asparaginyl endopeptidase (Sm32) for their activation from an inactive zymogen to fully active mature enzyme (Dalton et al., 1995, 1996; Brindley et al., 1997; Skelly and Shoemaker, 2001; Sajid et al., 2003; Delcroix et al., 2006; Krautz-Peterson and Skelly, 2008; Stack et al., 2011). Sm32 is also a cysteine peptidase, but is not a member of the papain superfamily (Dalton et al., 1995; Dalton and Brindley, 1996). Given their central importance in the biology of the parasite *S. mansoni* cysteine peptidases have been of interest as both vaccine candidates for disease prophylaxis and potential chemotherapeutic targets (Dalton and Brindley, 1996; Wasilewski et al., 1996; Abdulla et al., 2007).

In addition to their role in the biology of the parasite, we have shown that helminth cysteine peptidases have an ability to modulate the host immune response (O'Neill et al., 2000; Donnelly et al., 2010). Cysteine peptidases from such diverse sources as papaya (papain; Sokol et al., 2008), house dust mite (Derp1; Roche et al., 1997; Kikuchi et al., 2006), *Leishmania mexicana* (Pollock et al., 2003), and many fungal allergens (Shen et al., 1998; Kheradmand et al., 2002) have all been shown to skew the immune response toward a Th2 phenotype characterized by the release of IL-33 and IL-4 and the production of antigen-specific IgG1. Despite differences in amino acid sequence and tertiary structure, their shared ability to induce Th2 immune responses is dependent on their common enzymatic activity. Such is the potency of their ability to modulate immune responses that at low doses these cysteine peptidases can act as adjuvants, inducing Th2 responses to bystander antigens in the absence of another adjuvant (Chapman et al., 2007; Cunningham et al., 2012). Indeed, we have shown that the subcutaneous administration of papain prior to a challenge infection with *S. mansoni*, switched the parasite-specific immune response toward a Th2 phenotype. Furthermore, this approach resulted in a significant level of protection (50%) from infection (El Ridi and Tallima, 2013).

The mechanism by which cysteine peptidase enzymes drive the amplification of Th2 immune responses is a subject of vigorous research. It has been suggested that proteases may degrade intracellular epithelia cell proteins, or damage epithelial cells, for example in the lung, which respond by secreting stress-related cytokines including IL-12, IL-33, and thymic stromal lymphopoietin (TSLP), that subsequently activate mast cells and basophils (Tang et al., 2010; Liang et al., 2012). Innate lymphocytes, termed lung natural helper (LNH) cells, which produce IL-33 and TSLP, have also been implicated in papain-induced airway inflammation (Halim et al., 2012). Recent studies have indicated that a primary function of TSLP, produced as a result of protease activity, is to prevent the induction of Th1-inducing molecules such as IL-12 and CD70 from innate immune cells (Massacand et al., 2009), which in turn facilitates the development of Th2 immune responses. Interestingly, we found that, like papain, a vaccine formulation containing a combination of TSLP or IL-33 and larval ESP molecules reproducibly elicited production of parasite-specific Th2 cytokines and antibodies in response to a challenge infection with *S. mansoni*. Similar to the result achieved using papain, this vaccine formulation also elicited a highly significant ($P < 0.0001$) reduction of 62 (TSLP) to 78% (IL-33) in worm burden and worm egg load in host liver and small intestine (El Ridi and Tallima, 2013).

Indirectly, papain has been shown to mediate the differentiation of macrophages toward a Th2-associated M2 phenotype and via direct interaction, papain-treated macrophages are more likely to differentiate into M2 cells after stimulation with bacterial lipopolysaccharide, a ligand that more commonly induces inflammatory, Th1-associated M1 macrophages (Nhu et al., 2010, 2012). Consistent with these observations, we have previously reported that macrophages isolated from mice given a single parenteral injection of either SmCB1 or a *Fasciola hepatica* cathepsin L1 (FhCL1) were inhibited in their ability to produce Th1-inducing cytokines (Donnelly et al., 2010). The parasite proteases inhibited the TLR-TRIF dependent mechanism of activation by Th1-associated ligands and thus we proposed that these macrophages were more permissive to stimulation with Th2-promoting ligands. In the absence of TLR3-dependent signaling, production of Th2 cytokines is significantly increased during a murine infection of *S. mansoni* (Joshi et al., 2008).

Therefore, we suggested that, like papain, SmCB1 could act as an adjuvant to elicit an antigen-specific Th2 immune response to co-injected parasite molecules. However, we also proposed that as immunization with SmCB1 would result in the production of cathepsin B-specific antibodies, it would likely elicit a higher level of protection than papain. To that end, SmCB1 was administered subcutaneously to outbred mice, which were then challenged with an infection of *S. mansoni* cercariae. Consistent with the data seen for papain and in agreement with our hypothesis, highly significant ($P < 0.0001$) and reproducible reduction of 50–70% in challenge worm burden was achieved in five consecutive experiments. Protection was associated with predominance of Th2-related cytokines and antibodies (El Ridi et al., 2014). However, we found that the reduction in worm egg counts in host liver and small intestine was not as striking

as for the worm burden, in accordance with earlier findings correlating Th2 dominant responses with increased schistosome egg production (Wynn, 1999; Xu et al., 2009). Nevertheless, we observed that when a mixture of SmCB1 and FhCL1 were combined with the secreted proteins SG3PDH or SG3PDH and PRX-MAP a highly significant ($P < 0.0001$) and reproducible reduction of 66% in challenge worm burden and in worm egg load in liver and small intestine as well was achieved (El Ridi et al., 2014). The data confirmed that schistosome cysteine peptidases have in-built immune modulating properties that are protective on their own and have the potential to enhance the protective responses to other molecules. In line with all the evidence from allergen cysteine peptidases, this activity was related to their enzymatic activity since SmCB1 inactivated with inhibitors (E-64) or a non-active recombinant form of FhCL1 displayed markedly reduced level of Th2 mediators, and was associated with a significant decrease in protective capacity (El Ridi et al., 2014).

These results clearly demonstrate the induction of significant protection levels when an active parasite cysteine peptidase enzyme is used in the vaccine formulation with no additional requirement for an adjuvant. However, similar to the results achieved with the RA vaccination model, we have found inconsistencies between the characteristics of the antigen-specific T-cell response in protected animals. Instead, the key to the peptidase-mediated protective effect may be the stimulation of a particular type of antibody response. Mathematical models have concluded that in younger, untreated endemic human populations, parasite infections activate short-lived plasma cells that are essentially non-protective. In contrast, in older populations the cumulative deaths of infection worms, due to natural death or chemotherapy, releases an antigen load which stimulates a different immune response, characterized by the production of long-lived plasma cells which reduces worm load (Mitchell et al., 2012). While it is clear that cysteine proteases, irrespective of their source, clearly induce the production of Th2-type antibodies against themselves and bystander antigens, there has been no investigation into the nature of antibody-producing B-cell. Recently, it was reported that immunization of mice with low doses of a fish venom protease (Natterin), induced the differentiation of terminally differentiated, long-lived antibody-secreting cells and that this was dependent on the proteolytic activity of the natterin (Komegae et al., 2013). In addition, these authors demonstrated that the production of both IL-5 and IL-17 in response to the venom protease directly influenced the maintenance of the antibody secreting cells in the spleen (Grund et al., 2012). Therefore, its seems that the enzymatic activity of cysteine proteases, besides inducing antigen-specific cytokines, is also essential to generate survival signals necessary for the longevity of antibody secreting plasma cells.

Such potent, adjuvant-like effects of the cysteine peptidases may offer an innovative and feasible approach to developing a human vaccine formulation for protection against schistosomiasis. As we have learned, the delivery of parasite-secreted active cysteine peptidases alone or combined with other schistosome vaccine candidates, elicited levels of protection comparable to the bench-mark treatment of RA-cercariae. To date, the failure of many anti-schistosome vaccines has been attributed to the use of inappropriate adjuvants and/or delivery systems. Our data indicates that inclusion of active cysteine peptidases with in-built immunopotentiating activity in a vaccine preparation could preclude the need for a chemical adjuvant.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 17 February 2014; paper pending published: 25 March 2014; accepted: 17 April 2014; published online: 08 May 2014.

*Citation: El Ridi R, Tallima H, Dalton JP and Donnelly S (2014) Induction of protective immune responses against schistosomiasis using functionally active cysteine peptidases. Front. Genet. **5**:119. doi: 10.3389/fgene.2014.00119*

This article was submitted to Evolutionary and Genomic Microbiology, a section of the journal Frontiers in Genetics.