An historical and genomic view of schistosome conjugal biology with emphasis on sex-specific gene expression

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SUMMARY

The genetic programmes associated with the sexual biology of dioecious schistosomes remain a critically important but significantly understudied area of parasitology. Throughout the last four decades, progress has been slow in describing the gross antigenic and proteomic differences linked to sexually mature schistosomes and in characterizing some of the sex-associated transcripts and regulatory mechanisms induced during developmental maturation. These investigations have been severely hindered by the lack of complete EST/genomic information, as well as corresponding post- and functional-genomic tools for studying these pathogenic parasites. As near complete transcriptomes for *Schistosoma japonicum* and *S. mansoni* have recently been reported, and both DNA microarrays and post-transcriptional gene silencing have been applied to schistosomes, the tools and techniques for the high-throughput identification and characterization of transcripts involved in conjugal biology are now readily available. Here, an historical review is presented that summarizes some of the most significant findings associated with schistosome sex and sexual maturation during the last several decades. Following this discussion is a current overview of some modern day genomic approaches used to study schistosomes, which illustrates how major advances in the field of conjugal biology will be achieved.

Key words: Schistosome, sexual-biology, transcription, genomics, transcriptome, DNA microarray.

INTRODUCTION

Infection of vertebrate hosts with cercaria of the genus *Schistosoma* instigates the development of schistosomiasis, a debilitating disease that currently affects greater than 200 million people worldwide (Bergquist & Colley, 1998). While not all hosts suffer severe clinical manifestations associated with infection, it is clear that disease status is affected by multi-factorial phenomena including host genetics (Marquet *et al*. 1996; Dessein *et al*. 1999; Rodrigues *et al*. 1999; Henri *et al*. 2002), co-infection status (Marshall *et al*. 1999; Mwinzi *et al*. 2001; Mwatha *et al*. 2003; Naus *et al*. 2003), inappropriate host immune responses (Hoffmann, Wynn & Dunne, 2002*b*), age at infection (Chan *et al*. 1996), duration of infection (Mohamed-Ali *et al*. 1999), sex of infected individual (Booth *et al*. 2003) and many others. The development of pathological lesions during schistosomiasis has been extensively studied in laboratory animals over the years and the molecular machinery responsible is now being slowly unravelled and extrapolated to the human condition. Two seminal observations have revolutionized progress related to our advanced understanding of schistosome-induced pathology: (1) Warren's observations which established that the schistosome egg is responsible for the development of hepatic circumoval granulomatous inflammation during infection (Warren, 1978) and (2) Grzych *et al*.'s (Grzych *et al*. 1991) and Pearce *et al*.'s (Pearce *et al*. 1992) simultaneous finding that the schistosome egg is also responsible for driving type-2 immune response polarization during infection. Together, these important studies have directly linked the onset of schistosome-induced pathological reactions to the sexual maturation of worm pairs and the host's immunological response to egg deposition. Although not complete, recent follow-up studies from a variety of laboratories have elegantly elucidated many of the host's immunological and molecular determinants responsible for disease progression during infection.

Surprisingly, during this same period of time, very little progress has been made in describing the molecular and biochemical machinery responsible for parasite sexual maturation and egg production. Clearly, as egg production and host pathology are intimately associated, and egg dispersal is required for transmission of parasite gametes, the study of schistosome conjugal biology has not received the experimental attention it most definitely deserves. This review thus serves two major purposes: (1) to summarize some of the most important observations made over the last 30–40 years related to conjugal biology and the expression of sex-associated schistosome genes and (2) to describe how the application of parasite genomics, post-genomics and functional genomics will provide a much needed spark to fuel research into the understudied area of schistosome sexual maturation and egg development.

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BIOLOGY OF THE DIOECIOUS STATE

To successfully maintain a sexual life-cycle, schistosome parasites have evolved a life-style that involves the cooperation of morphologically distinct male and female forms (an excellent review of this subject has been provided by Basch (Basch, 1991)). Generally, males of the genus *Schistosoma* are larger, more muscular and responsible for holding the more slender female in a specialized structure called the gynaecophoral canal during their life-span together, which can be up to ten years in human populations (Fulford *et al*. 1995). At first glance, this dioecious life-style may appear inferior to the hermaphroditic ones employed by other parasitic trematodes (Digenea), where large amounts of genetic information can be maximally transmitted to the next generation simply by self-fertilization and without the need to find a mating partner. However, prolific asexual amplification in the intermediate snail host, along with efficient egg production in the definitive host more than compensates for the intrinsic problems associated with mate finding and serves to ensure dispersal of the genes responsible for heterozygosity within the genus. But several questions still remain: (1) when did schistosomes adopt a dioecious life-style and (2) what selective advantage does dioecy confer on these parasitic organisms? Although both questions are difficult to answer, several plausible hypotheses have been discussed related to the timing of dioecy acquisition. Amongst these, one hypothesis seems to have attracted the favour of most researchers and postulates that the transition from hermaphroditism to dioeciousness in blood flukes seems to have accompanied the acquisition of homeothermy by their definitive hosts (Basch, 1990). Expanding upon this tenet, Platt and Brooks have suggested that the colonization of homeotherms by vascular trematodes would have required precision egg-laying so that egg passage to the external environment was maximized and avoidance of host pathogenesis was minimized (Platt & Brooks, 1997). This scenario was likely accompanied by the selection of hermaphrodites that were either specialized for precision egg placement or generalized to maintain traits that would select for phenotypic males resulting in the origin of dioecious and dimorphic populations. Eventually, as the pressure to disperse eggs increased, more and more stress would have been placed on the specialized hermaphrodite to streamline its genome so that genes involved in efficient egg production and oviposition were optimally expressed – thus creating a phenotypic female. However, as male schistosomes are more likely to display hermaphroditic traits than females (Vogel, 1947; Short, 1948), the genes responsible for 'femaleness' have not been completely silenced in the genome of the male phenotype. In addition, the clasped (but not inseminated) female *S. mansoni* can produce viable, unfertilized, parthenogenetic, haploid eggs that yield miracidia capable of infecting *Biomphalaria* snails, which subsequently shed fully functional cercariae. Both facts attest to the genomic flexibility responsible for the maintenance of the life cycle under severe environmental circumstances (Basch & Basch, 1984). Therefore, it seems highly likely that as 'proto-schistosomes' broadened their definitive host specificity to homeothermic terrestrial vertebrates, they acquired a life style that best suited maximal egg dispersion with minimal host pathology. The formation of permanent pairs in a hostile host niche that mimics the hermaphroditic condition, along with the genomic flexibility to revert back to the ancestral state if need arises, has allowed schistosomes to become very successful and adaptable parasitic organisms.

But why evolve into stably-paired, sexually dimorphic individuals at all when hermaphroditism has proven to be, and still is, such a successful life style? Several possible reasons exist and include: (1) increasing genetic diversity in the gene pool; (2) minimizing genetic drift in the gene pool; and (3) dividing labour to maximize precision egg dispersal. Schistosomes from different genetic pools inhabiting the same host can cross fertilize each other and increase the genetic diversity of the species. However, hermaphroditic trematodes can effectively perform the same function by similar cross-fertilization events and thus this aspect of dioecy represents an important, but not critical driving force in schistosome evolution unless schistosome sex-specific genetic structures significantly increase offspring heterozygosity (Prugnolle *et al*. 2002, 2003). Conversely, long-term interactions of sexually distinct adult schistosomes from different gene pools could have been a driving force towards dioecy. This event would serve to minimize genetic drift by producing offspring expressing many (if not all) of the allelic combinations of their parents for the duration of conjugation, effectively driving genetic diversity among schistosomes. Finally, it is the separation of labour among sexually dimorphic individuals which probably represents one of the most important evolutionary advantages obtained through the acquisition of dioeciousness. Separation of labour allows the female schistosome to direct all of her genetic and metabolic machinery to maximize reproductive fitness and become a highly efficient egg-laying machine (Sturrock, 1966; Grant, 1971). The male schistosome ensures the survival of females (and of the species) by providing physical transportation within the vasculature (Basch, 1991), musculature to aid feeding (Gupta & Basch, 1987; Basch, 1990), and other chemo- or thigmatic-maturation factors (Robinson, 1960; Popiel & Basch, 1984) as well as providing sperm to fertilize the oocyte (which may not be necessary in some species

(Basch & Basch, 1984)). Schistosome functional dichotomy and labour division are two of the most studied areas of schistosome conjugal biology because of the implications these processes have on host pathology and egg transmission. Subsequently, the gene products that demonstrate sex-associated expression have been sought with the aim of identifying those that participate in the sexual maturation of the species as a means towards blocking host pathology and gamete transmission.

HISTORICAL STUDIES OF SCHISTOSOME CONJUGAL BIOLOGY

Schistosome sex is determined in the egg by the presence of the sex chromosomes Z and W (designation proposed by Atkinson (1980)). Individuals destined to be females contain the heterogametic ZW chromosomal pair and individuals predetermined to be males maintain the homogametic ZZ chromosomal combination. In addition to these sex chromosomes, differences in gene expression between gender may originate from any of the other 7 autosomal pairs of chromosomes maintained in the nuclei of these parasitic trematodes (Short, 1957; Liberatos & Short, 1983). Although some species of female schistosomes (e.g. *S. mansoni*) can produce viable eggs in the absence of insemination (Basch & Basch, 1984), male contact is absolutely required for ovary and vitelline gland development (Shaw & Erasmus, 1981; Shaw, 1987), and this interaction ultimately is linked to sexual maturation/gamete transmission within the genus (Moore, Yolles & Meleney, 1954). This may partially explain why there is often a male sex bias observed during experimental (Liberatos, 1987) and natural infections (Mitchell *et al*. 1990). Increased numbers of males enhance the chance of interacting with females to ensure sexual maturation and egg-laying. Many researchers have studied the phenomenon of male-induced female developmental maturation and have determined that this trait holds true regardless of the system utilized (*in vitro* or *in vivo*) or the species studied (*S. japonicum*, *S. mansoni*, *S. haematobium* and *S. bovis*) and interested readers are directed to the following reviews for further detailed information (Ribeiro-Paes & Rodrigues, 1997; Boag, Newton & Gasser, 2001). The exact mechanisms by which males direct female developmental maturation are currently unknown but may be related to transfer of some biomolecule (see Ribeiro-Paes & Rodrigues, 1997), physical massaging of the female to assist nutrient uptake and feeding (Gupta & Basch, 1987), induction of a sex-specific signalling cascade (Schussler, Grevelding & Kunz, 1997) or a combination of all three systems. This maturation signal exerts its effect on the female as long as there is physical contact between the two sexes regardless if the male is anorchid (Armstrong, 1965) or even dead (Popiel & Basch, 1984). An interesting caveat to these hypotheses regarding male-dependent developmental maturation can be found in experiments examining homosexual male pairing (Vogel, 1947; Armstrong, 1965). Here, the inner males appear to have developmental deficiencies, exactly the opposite of what would be expected if the outer male provides a general maturational signal to the enveloped partner (regardless of sex). There may exist a series of events that involves physical contact, exchange of some biomolecule and a subsequent signalling cascade that preferentially induces female sexual maturation during heterosexual pairing, and this series of events does not operate in a reciprocal or homosexual manner. Further insight into the molecular mechanisms of this interesting phenomenon will be discussed in further detail below.

IDENTIFICATION OF SEX-ASSOCIATED GENE PRODUCTS

Investigators wishing to study the compositional differences between male and female schistosomes in the 1970s–1980s had very little option as to how these experiments could be performed. As recombinant DNA techniques were just starting to be developed, differentially expressed male and female schistosome gene products were initially identified by high resolution SDS-polyacrylamide gel electrophoresis (Ruff, Davis & Werner, 1973; Ruppel & Cioli, 1977; Cordeiro & Gazzinelli, 1979; Atkinson & Atkinson, 1982; Aronstein & Strand, 1983, 1984; Braga, Tavares & Rumjanek, 1989). Despite the improvements offered by modern protein microsequencing and mass spectrophotometry technologies to identify fractionated polypeptides, these early studies contributed significantly to the understanding of schistosome conjugal biology and should be considered the forbears of modern schistosome proteomics. For example, Aronstein and Strand not only identified several female-specific glycoproteins expressed and released by *S. mansoni* (1 out of 30 proteins resolved were differentially released in the female culture medium), *S. japonicum* (4 out of 30), and *S. haematobium* (2 out of 30) (Aronstein & Strand, 1983), they subsequently identified differential host immunoreactivity against *S. mansoni* proteins obtained from males and females isolated from single-sex or bi-sex murine infections (Aronstein & Strand, 1984). Interestingly, some of the most striking gender- and species-specific differences observed were in the released glycoproteins. These proteins may be molecules destined for secretion and therefore may be representative of those subsequently identified by the modern signal sequence trap method (Smyth, *et al*. 2003). The proteomic studies performed during this era, along with those experiments utilizing monoclonal

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antibodies (e.g. Aronstein & Strand, 1985) provide a firm foundation for the continued study of gender-specific gene expression in schistosomes employing more modern techniques.

During the mid-1980s until the present day, identification of gender-associated gene products has been vastly accelerated through the utilization of recombinant DNA technologies. We now understand that, in a twenty-four hour period, a paired female schistosome converts the equivalent of almost her own body dry weight into eggs (Becker, 1977), produces several thousand vitelline cells to aid in egg-shell formation (Erasmus & Davies, 1979), and consumes approximately 8 million red blood cells (Lawrence, 1973). From these data, it can be proposed that female-specific gene products associated with the metabolic machinery of egg production and haemoglobin catabolism will be among those most highly represented during molecular biological studies of gender.

Identification of egg-shell transcripts and sex-associated regulatory factors

The use of recombinant DNA techniques to study schistosome biology has globally increased in the last 20 years and has led to the creation of many tools to aid the identification of gender-associated transcripts. Subtracted cDNA libraries made from mature female schistosome RNA pools (common male RNA elements subtracted) are one such tool and these libraries have been specifically utilized as a resource for identifying female-specific cDNA sequences (e.g. LoVerde, Rekosh & Bobek, 1989). As might be expected, egg-shell proteins are some of the most frequently identified female-associated gene products in these resources, having been characterized in *S. mansoni*, *S. japonicum* and *S. haematobium* (Bobek *et al*. 1986; Simpson & Knight, 1986; Johnson, Taylor & Cordingley, 1987; Bobek, Rekosh & LoVerde, 1988; Henkle *et al*. 1990; Chen, Rekosh & LoVerde, 1992). The developmental expression of these genes appears to be similar, as does their tissue-specific expression to vitelline glands of sexually mature females (Koster *et al*. 1988). Most of these egg-shell proteins are rich in basic amino acids (histidine and lysine) and contain many repeats of glycine and tyrosine (Bobek *et al*. 1988; Henkle *et al*. 1990). The structural importance of this amino acid bias has been examined (Rodrigues *et al*. 1989) and likely relates to the enzyme-dependent sclerotization reaction of trematode egg-shells, which will be discussed in further detail below. Furthermore, Chen *et al*. (1992) have subsequently estimated that the specific egg-shell genes encoding *S. mansoni* p14 and p48 contribute 5–10% and 0·3–0·5%, respectively, of the total mRNA pool in a mature female. Grevelding *et al*. have additionally demonstrated that p14 transcription in female parasites is rapidly down-regulated when separated from their corresponding male partners and again can be detected when re-pairing was initiated (Grevelding, Sommer & Kunz, 1997). Although this result confirmed that female sexual maturation is dependent upon a continued male interaction, it also importantly demonstrated that transcriptional regulation is intimately affected by such gender interactions. Further proof that transcriptional regulation is a driving force for egg-shell protein synthesis came in 1991 when Loverde and Chen (LoVerde, 1991) found conserved regulatory elements in the promoters of many egg-shell genes, including p14. These regulatory elements represent transcriptional binding sites (Shea *et al*. 1990) and appear to be essential, by similarity to homologous genes found in other species, for correct spatial and temporal expression (Mitsialis, Veletza & Kafatos, 1989). Additional proteins found in the vitelline gland (amongst other tissue types) that regulate egg-shell transcription are members of the nuclear retinoid X receptor (RXR) superfamily (Freebern *et al*. 1999*a*, *b*). Two family members, SmRXR1 and SmRXR2, have been demonstrated to bind cis-elements of the p14 egg-shell promoter in *S. mansoni* (Freebern *et al*. 1999*b*; Fantappie *et al*. 2001) and post-translationally controlled by Seven in Absentia (SmSINA (+)) mediated ubiquitination (Fantappie *et al*. 2003). Although SmRXR1 and SmRXR2 do not directly interact, each protein in capable of independently driving transcription in yeast-one-hybrid experiments further supporting their role as transcriptional activators in schistosomes. These data, as well as the identification of hormone regulatory elements in the 3′-UTR of another female associated gene transcript, F-10 (Giannini *et al*. 1995), strongly suggests that steroids/hormones may be intimately involved in the transcriptional regulation of female sexual maturation and egg-shell production. Studies further characterizing the molecular components of schistosome signal transduction have been performed with the female-associated expression of RAS, MAP and GAP kinases experimentally determined (Schussler *et al*. 1997; Kampkotter *et al*. 1999; Osman, Niles & LoVerde, 1999). Other schistosome signal transduction components such as SIP (McGonigle *et al*. 2001); Smad1/2 (Beall, McGonigle & Pearce, 2000), 14-3-3 (Schechtman *et al*. 2001; McGonigle, Loschiavo & Pearce, 2002), rab-related GTP binding protein (Loeffler & Bennett, 1996), SmTK4 (Knobloch *et al*. 2002) and Rho (Vermeire *et al*. 2003) have been molecularly identified and some gender-specific differences observed. The fine details of schistosome transcriptional regulation as it is related to sexual and developmental maturation are currently being examined and should prove to be an exciting area of research.

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The characterization of differential enzyme activities between sexually mature male and female schistosomes

Other female-associated gene products that are unrelated to the major egg-shell proteins have also been identified during the last 30 years. Numerous among these are enzymes involved in catabolism, sclerotization, purine salvage, neoglycoconjugate synthesis and detoxification of free gas radicals. Cathepsin B and L activity is higher in adult female parasite extracts than in adult male preparations (Dalton *et al*. 1996) and these endoproteinases have been localized to gut tissue where it is thought they aid in the digestion of haemoglobin. It has also recently been appreciated that other isoforms of these endoproteinases (SmCB2) exist and may show different functional, gender and tissue associations (Caffrey *et al*. 2002). An amidase has also shown female-associated expression and its localization to the gastrodermis suggests an additional role in digestive processes (Schussler *et al*. 1998). Together, the enhanced expression of cathepsins and amidases in the adult female digestive system is in line with increased ingestion of RBCs by this sex (Lawrence, 1973) and the need to catabolize this source of energy for distribution throughout the soma.

Phenol oxidase activity is also increased in adult females when compared to adult males (Seed & Bennett, 1980; Eshete & LoVerde, 1993; Fitzpatrick *et al*. 2004). This enzyme activity, found in vitelline glands (Bennett, Seed & Boff, 1978; Fitzpatrick *et al*. 2004), is likely involved in the sclerotization reaction of trematode egg-shells under the appropriate pH and calcium concentration conditions (Smyth & Clegg, 1959; Wells & Cordingley, 1991, 1992; Colhoun, Fairweather & Brennan, 1998). Here, tyrosine residues contained in egg-shell proteins are enzymically converted into DOPA quinones where they serve as effective substrates for nucleophilic attack by amino-rich lysine and histidine residues, also abundantly found in the same egg-shell proteins. The resulting phenol-oxidase catalyzed reaction (Seed & Bennett, 1980) induces a series of crosslinks between and within egg-shell polypeptides leading to the appearance of a 'hardened' egg-shell protecting the developing schistosome.

Another enzyme activity demonstrating female-specific association is adenylosuccinate lyase (ADL), the cDNA encoding this protein being first identified by Foulk *et al*. 2002). ADL is two times more active in adult females than males and is thought to participate in parasite nucleotide salvage as well as another, unidentified, female-specific function. Gender-associated expression of other nucleotide salvaging enzymes including adenylosuccinate synthetase, AMP deaminase, purine nucleoside phosphorylase and inosine-5′-monophosphate dehydrogenase await further investigation.

Tempone *et al*. (1999, 2002) demonstrated the increased transcription and enzymic activity of dolichol phosphate mannose synthase (SmDPMS) in female schistosomes and postulated that this enzyme was likely to be involved in the synthesis of glycoconjugates. It seems probable that females express greater quantities of SmDPMS than males as a means to help convert the increased amount of carbohydrates they acquire everyday (for egg-laying purposes (Becker, 1977)) into functional glycoconjugates.

A final enzyme demonstrating female-associated expression and activity is the selenium-containing phospholipid hydroperoxide glutathione peroxide (Roche *et al*. 1996). Although the expression of anti-oxidant genes is developmentally regulated in the more mature schistosome life-stages and thus postulated to play an important role in immune evasion (Mei & LoVerde, 1997), it is also likely that the increased expression of these genes has a gender-specific role. In particular, proteins with anti-oxidant properties may be involved in the detoxification of haemoglobin by-products, specifically $^{\bullet}O_{2^-}$ and hydrogen peroxide. Therefore, in addition to specialized schistosome immune evasion strategies, the expression of these genes may be linked to the mature female which needs to carefully metabolize, eliminate and store the bi-products associated with increased red blood cell consumption (Lawrence, 1973). As females assemble large quantities of haemozoin (a product which stores haeme in a non-toxic state) and also express greater quantities of ferritin-1 (protein which stores Fe (III) in a soluble and non-toxic form) (Dietzel *et al*. 1992; Schussler *et al*. 1995), this hypothesis seems plausible. In support of this hypothesis, an extracellular superoxide dismutase (Ex-SOD) homologue was recently found to be enriched in adult female schistosomes and the activity of this enzyme postulated to neutralize the build-up of toxic $^{\bullet}O_{2^-}$ during haemoglobin digestion (Fitzpatrick *et al*. 2004). Further work determining the gender-specific expression patterns of all anti-oxidant enzymes would help clarify the specific role each plays during schistosome developmental maturation processes.

Additional gene products expressed by sexually mature female schistosomes

Other differentially expressed genes in the mature female include the fs800 protein (Reis *et al*. 1989) and a mucin-like polypeptide (Menrath, Michel & Kunz, 1995). Although no functional homology can currently be assigned to the two ORFs encoding the fs800 protein, *in situ* localization suggests that these transcripts are localized to the vitelline glands of mature female parasites. Also based on *in situ* hybridization localization to the ootype entrance, the mucin-like polypeptide has been postulated to

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function as a protective molecule for the reproductive tract and in an inhibitory fashion to prevent premature egg-shell synthesis. Further characterization of these two molecules may uncover a specific functional role in female sexual maturation.

SCHISTOSOME GENOMICS, POST-GENOMICS AND FUNCTIONAL GENOMICS

It has been the combined vision of many schistosome researchers that has made the sequencing of this important pathogen a reality. Although the entire genomic complement (introns, promoters, etc.) has yet to be revealed, almost all of the transcriptome from both *S. japonicum* and *S. mansoni* has recently been identified (Hu *et al*. 2003; Verjovski-Almeida *et al*. 2003). With the addition of whole genome shotgun projects, initiated at both TIGR (the Institute for Genomic Research, USA) and the Wellcome Trust Sanger Institute (UK), it is expected that 8X coverage of the *S. mansoni* genome soon will be obtained (http://www.sanger.ac.uk/Projects/S_mansoni/) and will provide previously unknown and important structural information. Together, EST and these genomic projects present an opportunity for researchers interested in schistosome conjugal biology to accelerate their studies at a startling rate by capitalizing on the deposited sequence information to use in post- and functional-genomic activities. For purposes of this review, the post-genomic activity discussed here involves the global analysis of mRNA expression using DNA microarrays, whereas the functional-genomic activity examined comprises post-transcriptional gene silencing of mRNAs by use of RNA interference. How these techniques can and will be applied to schistosome conjugal biology will be presented.

Schistosome genomics

Recently, two important papers have been published which describe, to a large degree, the complete EST complement of both *S. japonicum* and *S. mansoni* (Hu *et al*. 2003; Verjovski-Almeida *et al*. 2003). Taking advantage of multiple cDNA libraries derived from diverse parasite life-stages, the authors of these two genomic studies have made available through public databases (GenBank), approximately 13 131 *S. japonicum* and 30 000 *S. mansoni* unique gene clusters (represented as singletons and contigs). One aspect of both studies was the assignment of transcripts more abundantly represented in each of the starting cDNA libraries and thus the authors hypothesized that these sequences correspond to stage- or sex-specific molecules. While correlations made between number of EST reads for any given transcript and differential expression cannot always be inferred (Ajioka *et al*. 1998), at the very least, the authors of these two seminal manuscripts have provided new lists of potential gender-associated molecules available for future studies into schistosome conjugal biology. More importantly, the availability of near complete transcriptome information for each of these important pathogens provides the structural elements (sequence information) absolutely essential for the successful implementation of both post- and functional-genomic activities.

Schistosome post-genomics

The development of DNA microarray technology (Schena *et al*. 1995; Shalon, Smith & Brown, 1996) to interrogate differential gene expression between two different pools of RNA has made this post-genomic application attractive to investigators wishing to profile transcriptional differences between adult male and female schistosomes. With the recent identification of nearly every open reading frame for both *S. japonicum* and *S. mansoni*, genome-wide gene expression profiling of these important pathogens will soon be an experimental reality. Towards this end, two pilot studies were recently performed that illustrated the power of DNA microarray technology in reproducibly detecting sexually mature male and female transcripts in *S. mansoni* (Hoffmann, Johnston & Dunne, 2002*a*) and *S. japonicum* (Fitzpatrick *et al*. 2004). To identify gender-associated gene expression profiles in *S. mansoni*, a cDNA microarray was fabricated from a small collection (representative of the *S. mansoni* EST database at the time) of ESTs derived from three different cDNA libraries and two different developmental stages. The importance of this study was that it not only provided a list of female-associated gene transcripts, but it also yielded information related to the expression of male-associated genes, a subject that has received little experimental attention over the years. Briefly, Hoffmann *et al*. (2002*a*) reported the identification of 12 new female-associated and 4 new male-associated gene transcripts in the mature adult schistosome. Although some of these transcripts contained NCBI database homologues (female-associated transcripts: tyrosinase (AI111005); 60S ribosomal protein L12 subunit (R95618); and fs800 protein homologue (N21956) – male-associated transcripts: dynein light chain 3 homologue (N21858); and tropomyosin (R95521, R95617, R95512)), many of the new associations were not significantly similar to any database entry presumably due to the length of the EST sequence being compared. However, recent BLASTn analysis of the differentially expressed EST elements using the newly annotated *S. mansoni* EST dataset (http://cancer.lbi.ic.unicamp.br/cgibin/schisto6/blast/form.pl) revealed some novel observations not originally reported by Hoffmann *et al*. 2002*a*). These new observations relate to the

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female-associated expression of a *Plasmodium* histidine-rich protein homologue (AI111039), a *Clonorchis sinensis* egg protein homologue (AI110935), and a putative tumour suppressor (AA559404) as well as the male-associated expression of a *Drosophila melanogaster* extracellular matrix protein homologue (AI111017). Some of the possible interpretations related to these differential gender-associated gene transcriptional profiles have been discussed (Hoffmann *et al*. 2002*a*) but, in light of the new database homologies, can be revisited.

The DNA microarray identification of tyrosinase as being differentially expressed in the female clearly confirms the enzymatic studies for phenol oxidase activity as performed by Seed, Kilts & Bennett (1980) and Eshete & LoVerde (1993) and suggests that this gene product is associated with the important process of egg-shell sclerotization (Hoffmann *et al*., unpublished observations). The newly annotated histidine-rich protein (HRP) also identified in this DNA microarray study could either participate in egg-shell development (histidine amino groups participating in nucleophilic attack of tyrosinase-converted DOPA-quinone groups) or haemozoin pigment formation as observed for HRPs in *Plasmodium* (Pandey *et al*. 2003). What role the *C. sinensis* egg protein homologue plays in egg development is unknown but it represents one of the only schistosome egg proteins thus far identified that do not share sequence similarity to the major egg-shell polypeptides. Therefore, this protein may be unrelated to egg-shell formation but critical for some other aspect of egg developmental biology. The female-associated expression of a tumour suppressor homologue is interesting and its gender-associated biological implications await further investigation.

Some of the male-associated transcripts identified in this DNA microarray study represent common components of the tegument (tropomyosin and dynein light chain 3) but the localization and function of the extracellular matrix protein homologue remains unknown. It is tempting to speculate that this protein serves in some adhesive function on the surface of male schistosomes, similar to that proposed for SmGCP (Bostic & Strand, 1996) and, therefore, this extracellular matrix protein homologue may participate in some aspect of male/female interactions during pairing.

In another set of related studies, Fitzpatrick *et al*. (2004) have recently designed a *S. japonicum* cDNA microarray from 457 ESTs derived from three different life-stages in order to specifically characterize sexually mature adult gene expression profiles of two related Chinese strains (Anhui and Zhejiang strains). The results of this study provided additional male- and female-associated transcripts linked to sexual maturation, egg production and tegumental biology and will provide the basis for future functional investigations within this schistosome species. Importantly, many of gender-enriched transcripts in this schistosome species were found to be similar between strains and additionally confirmed those results first reported in the DNA microarray study of *S. mansoni* gene transcription. Specifically, in addition to identifying female enriched Ex-SOD expression, Fitzpatrick *et al*. (2004) also identified the female associated expression of two distinct tyrosinase homologues (SjTYR1 and SjTYR2), a large blood-brain barrier amino acid transporter, two histidine-rich proteins, and acetyl-CoA acetyltransferase homologue and several other unique transcripts. Further work is needed to understand the importance of these genes' transcriptional bias. The male-associated expression of actin, myosin-regulatory light chains, dynein light chain 3, TMSF4, Sj25 and others uncovered in this study also provide additional transcriptional evidence for labour division amongst the schistosomes and suggest the male's penultimate role in dioecy may be mechanical support.

From these first successful studies utilizing DNA microarrays to identify gender-associated gene expression in schistosomes, an expanded *S. mansoni* microarray has recently been developed for use in further developmental maturation studies. In a continuing series of experiments, Hoffmann *et al*. (unpublished observation) have fabricated an oligonucleotide DNA microarray consisting of ~50% of the *S. mansoni* transcriptome complement (~7000 unique sequences). This resource is currently being used for identifying stage- and sex-associated metabolic pathways, gene transcripts associated with the development of gender and exons differentially utilized amongst a series of well characterized genes. Presently, use of these DNA microarrays has lead to the identification of 140 female-enriched transcripts and 86 male-associated molecules in the mature schistosome (Hoffmann *et al*., unpublished). Continued use of these schistosome-specific DNA microarrays will help refine the way conjugal biology is studied and lead to an increased understanding of gender-specific and developmental gene expression.

Schistosome functional genomics

Techniques to characterize the function of any identified gender-specific transcript are limited to heterologous or homologous-based systems (Boyle & Yoshino, 2003). Unfortunately, for helminths these techniques are not widely established, are difficult to perform or simply are in their infancy. One particular technique, however, that has showed promise for the characterization of gene function in a homologous system is the use of post-transcriptional gene silencing (PTGS) mediated by double-stranded RNA (dsRNA) (Hannon, 2002). This technique has recently been adapted to schistosomes and was

effective in 'knocking down' the expression of cathepsin B activity during the transformation of cercariae to schistosomula (Skelly, Da'dara & Harn, 2003) and the expression of a glucose transporter (SGTP1) during the transformation of miracidia to sporocysts (Boyle *et al*. 2003). Both of these studies involved soaking larval stages in the presence of dsRNA for the gene of interest during an experimental procedure that induced extensive parasite surface membrane rearrangement and turnover. The authors of both studies hypothesized that experimentally-induced membrane rearrangement was critical for the induction of schistosome PTGS as this technique allowed entry of dsRNA into the schistosome and led to the observed 'knock down' effect. Currently there is no evidence to suggest that adult parasites cannot be manipulated in this fashion; however, attempts thus far have been less than effective. More investigators working in this area, as well as in the area of parasite transfection technologies, will eventually lead to a resolution of this problem, and soon PTGS may be available for all stages of schistosome development leading to a high-throughput means to assess gene function.

This technique would greatly benefit the functional characterization of genes identified during conjugal biology studies. Here, the role of any gene transcript associated with the process of sexual maturation, gender association, egg production or developmental biology could be identified and placed in the larger context of disease pathogenesis during schistosomiasis. For example, it could be envisioned that studies soon will be conducted involving the *in vitro* manipulation of schistosomes by PTGS followed by direct implantation of these genetically-modified organisms into their hosts. The specific effect of such gene manipulation on host pathology and gamete transmission would thus be experimentally determined. From these experiments, novel chemotherapeutic or immunoprophylactic targets could be readily identified, which in turn, would lead to strategies useful for combating schistosomiasis.

CONCLUSION

Current molecular and genomic techniques present the opportunity for identifying and functionally characterizing most gender-specific transcripts associated with schistosome conjugal biology, a feat that has not been possible at any time during the history of studying this pathogenic organism. We have learned from historical studies of sex, sexual maturation and male-female interactions that schistosomes are incredibly complex and evolutionarily-gifted parasites that depend upon dioecy to propagate gamete transmission effectively. It is now feasible to unravel some of the hidden complexities involved in schistosome conjugal biology and gender-associated gene expression by utilizing EST and genomic sequencing efforts to firmly establish high-throughput post- and functional-genomic techniques. In combination with established experimental animal models and *in vitro* culturing systems, these modern technological advances will logarithmically increase knowledge related to schistosome sexual biology and lead to the resolution of some of this parasite's fascinating and enigmatic secrets.

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