Cell. Mol. Life Sci.

DOI 10.1007/s00018-005-5578-1

Cellular and Molecular Life Sciences

Research Article

Transcriptome profiling of lung schistosomula, *in vitro* cultured schistosomula and adult *Schistosoma japonicum*

M. Chai[a], D. P. McManus[a], R. McInnes[b], L. Moertel[a, c], M. Tran[a], A. Loukas[a], M. K. Jones[a] and G. N. Gobert[a, *]

[a] Queensland Institute of Medical Research and Australian Centre for International Health & Nutrition, 300 Herston Road, Brisbane, Queensland 4029 (Australia), Fax: +61 7 3362 0104, e-mail: geoffG@qimr.edu.au
[b] Agilent Technologies, 347 Burwood Highway, Forest Hill, Victoria 3131 (Australia)
[c] The School of Chemical and Biomedical Sciences, Central Queensland University, Bruce Hwy, North Rockhampton, Queensland 4702 (Australia)

Received 6 December 2005; received after revision 19 January 2006; accepted 3 February 2006

Abstract. The schistosomulum is the main target of vaccine-induced protective immunity; however, most studies have utilized schistosomula produced by mechanical transformation of infective larvae followed by *in vitro* culture rather than larvae isolated directly from the lungs of infected mammals. Using transmission electron microscopy, we demonstrated that there was little difference in the ultrastructure of *Schistosoma japonicum* schistosomula obtained by the two methods. However, significant differences in gene expression profiles were apparent when we used an oligonucleotide microarray to compare the gene expression profiles of schistosomula obtained *in vivo* from lung tissue with those maintained *in vitro*, and with adult worms of *S. japonicum*. It is likely that host environmental factors, which cannot be reliably reproduced *in vitro*, do influence the growth, development and overall biology of schistosomes. Thus caution is urged when using *in vitro*-cultured schistosomes and mechanically transformed/cultured schistosomula in molecular, biochemical and immunological studies.

Keywords: Microarray, *Schistosoma japonicum*, schistosomula, gene expression, culture conditions.

Introduction

Schistosomiasis is a chronic parasitic disease caused by digenean blood flukes with more than 250 million people in 75 countries affected, and a further 600 million people at significant risk of infection [1, 2]. Schistosomiasis has not decreased significantly over the last 50 years, despite concerted control efforts in many endemic areas [3, 4]. No anti-schistosome vaccines are available and, although there is an effective drug, praziquantel, the possibility of wide-scale drug resistance developing is a major concern. Novel therapeutic interventions for the treatment and control of schistosomiasis are urgently needed.

* Corresponding author.

Two releases of data for *S. japonicum* [5] and *S. mansoni* [6] added approximately 160 000 new schistosome expressed sequence tags (ESTs) to GenBank. These two studies [5, 6] resulted in the clustering of ESTs of assembled sequences, which were used to design the oligonucleotide microarray described in this report. The microarray consists of 22 575 features (including 3354 controls), making this the largest microarray used to date for the analysis of the schistosome transcriptome. The 19 221 target sequence probes on the microarray include 12 166 probes derived from *S. mansoni* contigs, and 7055 probes derived from *S. japonicum* contigs. The use of microarrays in parasitology is a growing field of research [7].

All *Schistosoma* infections follow direct contact with fresh water cercariae, which penetrate the skin of humans, and shed their bifurcated tails. The resulting schis-

tosomula enter capillaries and lymphatic vessels en route to the lungs. After several days, the worms migrate to the portal venous system where they mature and pair. Few studies of the ultrastructure, metabolism, immunogenicity or gene expression profiles of schistosomula have been reported, and, in these, the larvae were artificially transformed and cultured *in vitro* [8–13]. Due to their relative ease of isolation and obvious importance to the human host, the biology of the cercariae and adult parasites has been more widely investigated than schistosomula. The transient lung stage of schistosomes, the schistosomulum, is the main target of vaccine-induced protective immunity [4]. The current study is unique in that it reveals previously unreported differences in the gene expression profiles of *in vitro*-transformed and cultured schistosomula compared with larvae that have penetrated skin and migrated to the lungs *in vivo.*

Materials and methods

Parasites. *Oncomelania hupensis hupensis* snails, infected with the Chinese mainland strain (Anhui population) of *S. japonicum,* were imported from China as required (kindly provided by Professor Guo Jiagang, National Institute of Parasitic Diseases, CDC, Shanghai). Cercariae were shed by placing snails in deionized water and exposed to incandescent light for 3–4 h. Cercariae, collected from the water surface by a bacteriological loop, were used to infect outbred Swiss mice.

Microarray composition. Oligonucleotide microarray design was based on two extensive schistosome EST databases: *S. japonicum* EST database (http://*Schistosoma*.chgc.sh.cn/), and *S. mansoni* Gene Index (http://www.tigr.org/tdb/e2k1/sma1/).

The final microarray design consisted of 22 575 features (including 3354 controls), making this the most comprehensive oligonucleotide DNA microarray available for the analysis of the schistosome transcriptome. The 19 221 60-mer probes on the microarray include 12 166 probes derived from *S. mansoni* contigs and 7055 probes derived from *S. japonicum* contigs, effectively providing a broader coverage of the majority of the schistosome transcriptome. The complete layout of the *Schistosoma* microarray is presented in Supplementary Table 5. Contained in this master file is the designated contig number, probe position on the microarray, assembled sequence used to design the 60-mer probe, known annotation and any associated gene ontologies (GO). Probes designed for the *S. mansoni* EST clusters were designated TC####, while *S. japonicum* derived probes were designated Contig####. Annotation and GO were provided by the *S. mansoni* and *S. japonicum* EST databases, respectively. The use of a microarray from two closely related species, while allowing a more powerful tool, does require care in the interpretation of results. Any sequence divergence between the species could affect hybridization efficiencies, but since in this current study we are using mRNA from one species (one transcriptome) only, and comparing different life cycle stages within that species, this is not an issue here. Since any homology variation between the probe of one species and the target mRNA pool will be the same in both target samples then hybridization efficiencies will be the same.

Microarray design and raw data has been submitted to MIAMExpress at European Informatics Institute, Hinxton, UK (http://www.ebi.ac.uk/miamexpress/).

Real-time PCR confirmation of microarray data. A subset of genes from the array representing genes that were over-expressed, under-expressed and equally expressed, were quantified using real-time PCR as an independent measure of relative gene expression (Table 1). Real-time PCR is a technique that uses fluorescent labels to monitor the accumulation of PCR product during the log phase of the reaction, allowing quantification relative to a co-amplified reference gene (in this case, GAPDH) [14]. From microarray results, it was noted that GAPDH does vary in gene expression between the three life cycle stage examined. However, in all three cases GAPDH was expressed at high levels (as indicated by signal intensity) and was a good example of an endogenous, if not strictly a 'housekeeping', gene [15]. GAPDH was not used to normalize any of the results from real-time PCR. Examples of genes that were notably differentially expressed in all microarray hybridizations include: Contig2397, *S. japonicum* Sj-Ts5 mRNA, complete cds; Contig8330, *S. japonicum* calpain mRNA, partial cds; TC16685, weakly similar to cysteine string protein (CCCS1), Pacific electric ray, partial (31%); and TC9024, similar to 'Similar to heat shock 60kDa protein 1′ (chaperonin), *Xenopus laevis*, partial (62%).

Although the RT-PCR results consistently confirmed results for directionality of regulation, fold changes, as calculated by RT-PCR, showed considerable variance and were consistently greater than fold changes observed in the microarrays. In addition to amplified RNA of adult schistosomes, lung schistosomula and mechanically transformed schistosomula, quantification of gene expression by RT-PCR was carried out on the unamplified mRNA of adult parasites to test for bias in the amplification procedure. While, results indicated that genes were disproportionately amplified during the amplification of mRNA using the Amino Allyl MessageAmp™II aRNA Kit (data not shown), all target RNA used in the microarrays underwent the same amplification procedure from identical starting amounts of mRNA.

Additional details of material and methods are available as a supplementary document outlining parasite methods, transmission electron microscopy protocols microarray design, annotation and analysis methods.

Results and discussion

Comparative ultrastructure of schistosomula obtained *in vivo* and *in vitro*

The ultrastructure of lung stage schistosomula (3 days post infection) of *S. japonicum* obtained *in vivo* (IVS: *in vivo* schistosomula) was compared with that of mechanically transformed, and then 3-day *in vitro*-cultured, motile schistosomula (MTS: mechanically transformed 3-day-cultured schistosomula). Transmission electron microscopy was used to examine the tegument and associated structures, cell types of the underlying parenchyma and various organ systems. General ultrastructural features of the tegument matrix, underlying muscle, sub-tegumental cells and parenchymal cells were similar between IVS and MTS parasites. The predominantly heptalaminate tegument was 500–700-nm thick and extensively pitted, often organized into sagittal ridges (Fig. 1a). The apical surface of MTS was covered in regions with a 1–200-nm thick electron-dense fibrous layer reminiscent of the glycocalyx of the cercarial stage [16], which was not present in IVS. Prominent tegumental spines were present and the basal lamina consisted of sheet-like invaginations of the basement membrane extending into the tegument matrix. Cytoplasmic bridges were seen joining sub-tegumental cell bodies in the underlying parenchyma to the tegument matrix (Fig. 1b). Cytoplasmic inclusions in the tegument included elongated bodies and large membranous bodies (Fig. 1c, d).

The structures of the cecum of IVS and MTS were very similar to that of the adult parasite of *S. mansoni* [17–19]. The cecum of the *in vivo* parasite consisted of a syncytial gastrodermis approximately 600 nm in thickness, with thicker projections into the lumen, which appeared to trap gut contents including electron-dense granulated material and lipid droplets (Fig. 2a). This interaction of the gut syncytium with lipid droplets and other gut contents was very similar to that described in the active cecum of adult *S. mansoni* [19].

Parallel folds of the apical surface convoluted the surface of the esophagus were observed in both IVS and MTS parasites (Fig. 2b), creating an undulating surface with crypts in which luminal contents were partially isolated. The proximal esophageal lining resembled the outer tegument of the parasite, featuring numerous laminated vesicles and elongated bodies in the matrix. The distal esophagus narrowed forming a demarcation at the junction with the cecum, as previously described in adult *S. mansoni* [19].

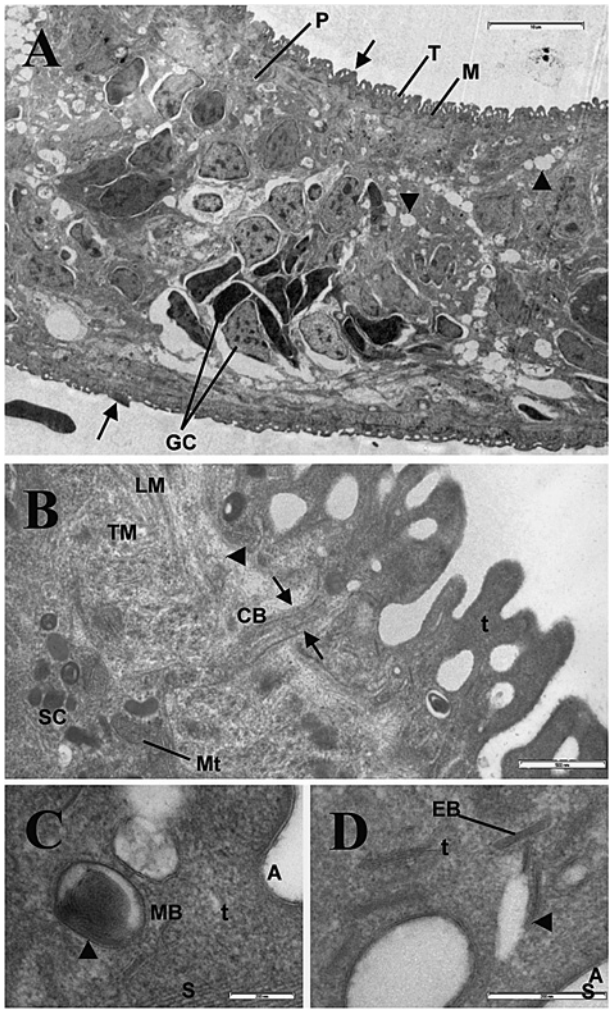

Figure 1. Transmission electron micrographs of schistosomula obtained directly from the lungs of mice. (*A*) Lung schistosomulum cut in longitudinal section. Spines (arrows) are visible projecting through the tegument (T), and muscle (M) can be seen underlying the tegument. Nested groups of germinal cells (GC) can be seen. Lipids (arrowheads) can be seen embedded in the parenchymal matrix (P). Scale bar = 10 µm. (*B*) Micrograph of a lung schistosomulum containing a cytoplasmic bridge (CB). The boundaries of the cytoplasmic bridge are indicated by arrows. The bridge passes through transverse (TM) and longitudinal muscle (LM) to connect a subtegumental cell (SC) to the tegument matrix (t). Mitochondria (Mt) and various organelles can be seen at the juncture of the subtegumental cell to the cytoplasmic bridge. An invagination of the basement membrane of the tegument is indicated with an arrowhead. Scale bar = 500 nm. (*C*) The tegument matrix (t) is limited by a multilaminate apical membrane (A). A membranous body (MB) containing partially extracted concentric laminations (arrowhead) is indicated. A spine (S) with paracrystalline actin array can also be seen. Scale bar = 200 nm. (*D*) Elongated bodies (EB), several of which are apparently merging (arrowhead) with an invagination (viewed in cross section) of the apical membrane (A) of the tegument matrix (t). Scale bar = 200 nm.

Comparison of gene expression in lung stage schistosomula and adults

Combined and filtered data revealed 3777 differentially expressed genes with p values of ≤0.001. Of these, 1759 were over-expressed in adult worms relative to lung stage schistosomula, and 2018 were over-expressed in lung schistosomula relative to adult schistosomes, the distribution of the genes is presented in a scatterplot in Fig. 3a. The percentage of major GO categories present within these differentially expressed genes is presented in Fig. 4. Genes over-expressed in the adult parasite contained 197 (1761

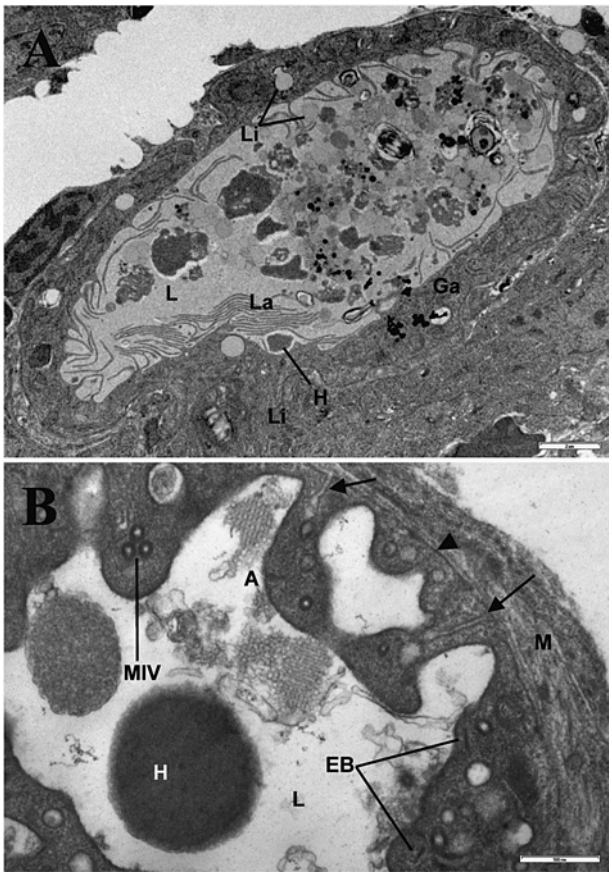

Figure 2. Transmission electron micrographs of the gut and esophagus regions of the lung schistosomula. (*A*) The cecum of the lung stage schistosomulum containing lamellae (La) of thickenings of the gastrodermis (Ga) sequestering luminal contents such as heme-like material (H). Lipids (Li) can be observed within the lumen (L) and also within the gastrodermis. Scale bar = 2 µm. (*B*) The esophagus of a lung schistosomulum in cross-section with arrowheads indicating the basal membrane, which frequently invaginates (arrows) toward the lumen (L). The ground substance of the esophagus contains elongated bodies (EB) and multilaminated vesicles (MlV). Various luminal contents can be observed. Electron-dense material (H) may potentially be heme or other products of blood digestion. The apical membrane (A) undulates. A layer of muscle (M) surrounds the esophagus. Scale bar = 500 nm

individual GO categories) genes with an associate GO, while the 126 (2022 individual GO allocations categories) of the genes that were over-expressed in IVS had associated GO. Complete lists of the differentially expressed genes are presented in Supplementary Tables 1 and 2.

A subset of genes (9) was randomly selected for further validation by real-time PCR (Table 1). Although the real-time PCR results consistently confirmed results for directionality of regulation, fold changes as calculated by real-time PCR showed variance and were consistently greater than fold changes observed in the microarrays, an observation common in microarray experiments [20]. To test for bias in the amplification of RNA, real-time PCR of amplified RNA of adult schistosomes and unamplified mRNA of adult parasites were compared. Differences in gene expression levels were apparent between mRNA isolated directly from adult parasites and mRNA that had been subsequently amplified, a phenomenon that had been mentioned in the manual of the amplification kit used (Amino Allyl MessageAmp™II aRNA Kit Ambion). The kit does mention that any bias in the amplification method is due to the size and sequence of individual gene products and is not a random event. All of the mRNA used in this study for microarray hybridization were amplified, but were subjected to a prior BioAnalyzer analysis to check the fidelity of the starting material, ensuring that no variable degradation of the initial staring mRNA could influence the final amplified product. All target RNAs used in the microarrays underwent the same amplification procedure from similar starting amounts of mRNA.

Enzymes involved in hemoglobin digestion are over-expressed in adult schistosomes. Adult schistosomes acquire amino acids by sequentially degrading hemoglobin from ingested host erythrocytes using a number of proteinases [18, 21]. These include endopeptidases such as cathepsins B, D and L, and exopeptidases such as cathepsin C [22]. Two probe sequences designed to bind different regions of the transcript for *S. japonicum* cathepsin B, are present on the custom microarray (Contig7921 and Contig8839), and were over-expressed in adult worms (Table 2) with fold changes of 10.71 (p = 5.22E–19) and 2.52 (p = 1.81E–07). This variable fold-change between different probes for the same gene may be indicative of cross-hybridization to an unreported homologue. Probes designed to *S. japonicum* cathepsin L precursor, preprocathepsin L (Contig8520) and a *S. japonicum* clustered sequence (Contig8499) homologous to *S. mansoni* cathepsin C, showed up-regulation in the adult parasite with a fold change of 1.65 (p = 0) and up-regulation in the adult parasite with a fold change of 1.8 (p = 3.59E–06). An increased abundance of transcripts for hemoglobin digestion in adults suggests an enhanced role for these enzymes compared with lung schistosomula. The expression of these genes in lung schistosomula supports the ultrastructural observations that lung schistosomula have a functional gut.

Genes involved in glucose transport are over-expressed in adult schistosomes. The mechanisms by which lung schistosomula obtain nutrients via the tegument or by ingestion of host red blood cells remains an unresolved issue. It is clear that adult schistosomes absorb host glucose across their hydrophobic surface membranes using integral membrane proteins called schistosome glucose transporter proteins (SGTPs) [23]. SGTP1 and SGTP4 have been localized to the basal and apical

Table 1. Selected microarray transcript data validated by real-time PCR analysis for lung schistosomula compared with either adult or mechanically transformed/*in vitro*-cultured schistosomula.

Contig	Fold change from microarray*	p value	Mean fold change (real-time PCR)
Adults/IVS			
Contig8873	–1.10	0.10	–2.96
Contig6432	–1.25	0.03	5.35
Contig6953	53.03	1.86E–39	75.90
Contig2733	–2.63	1.51E–16	–2.70
TC11261	–2.00	3.02E–06	1.43
TC15532	1.85	2.80E–10	7.78
Contig2522	2.41	5.73E–06	12.54
MTS/IVS			
Contig6953	–2.75	5.75E–09	–8.50
Contig2522	9.41	3.26E–44	12.54

* Fold changes are expressed as the ratio of gene expression in adults/lung schistosomula or mechanically transformed/cultured schistosomula/lung schistosomula.

Table 2. Selected genes involved in nutritional metabolism that were over-expressed in the adult parasite or genes involved in immune evasion and the stress response under expressed in adult *S. japonicum*.

Gene	Probe	Fold*	p value
Metabolism and over expressed in adult			
Homology to *S. mansoni* glucose transporter protein (SGTP1)	Contig8527	3.8	3.19E–07
S. japonicum cathepsin B endopeptidase	Contig7921	10.71	5.22E–19
S. japonicum cathepsin B endopeptidase	Contig8839	2.52	1.81E–07
S. mansoni cathepsin C	Contig8499	1.8	3.59E–06
S. japonicum preprocathepsin L	Contig8250	1.65	0
C. albicans serine/threonine protein kinase gene	Contig2944	1.71	1.21E–09
S. mansoni tryptophan hydroxylase	Contig5573	1.93	3.19E–22
Xenopus laevis similar to adenylosuccinate synthetase 2	Contig7465	3.21	0
Cathepsin B endopeptidase [44]	TC10560	8	2.55E–14
Protein glycotransferase {*Branchiostoma belcheri tsingtaunese*}	TC10667	1.98	1.45E–23
Homologue to peptidase M 2 {*S. japonicum*}	TC10827	2.69	4.02E–04
O-sialoglycoprotein endopeptidase {*Homo sapiens*}	TC15335	3.84	0
Immune evasion and under-expressed in adult			
S. mansoni anti-inflammatory protein (Sm16)	Contig7271	–8.58	0
Homology to both *S. japonicum* muscle paramyosin and *S. japonicum* paramyosin mRNA	TC16824	–8.93	3.62E–37
Homology to *S. mansoni* HSP70	Contig6517	–3.46	3.08E–25

* Fold changes are expressed as the ratio of gene expression in adult schistosomes (Cy5)/lung schistosomula (Cy3).

membranes of the tegument of adult schistosomes and *in vitro*-transformed schistosomula of *S. mansoni* [24]. Two probes (Contig8527 and Contig4074) against clustered *S. japonicum* sequences, homologous to *S. mansoni* SGTP1 and SGTP4, were studied, and Contig8527 was shown to be over-expressed 3.8-fold in the adult parasite ($p = 3.19E–07$), indicating an increased role for SGTP1 in this stage compared to the lung schistosomulum (Table 2).

Genes involved in fatty acid transport are over-expressed in adult schistosomes. Similarly, fatty acids derived from the definitive host are required in larger amounts during the adult stage for egg production [25]. This is demonstrated by the over-expression of a gene encoding a fatty acid binding protein (FABP) in the adult parasite compared to the lung schistosomula stage. Fold changes of 6.35–48.56 were observed in the adult parasite from two probes with homology to FABP (Contig1965, 6.35, $p = 0$; and Contig8946, 48.56, $p = 0$). The fatty acid requirements of lung schistosomula can also be assumed to be great as the parasite grows in size, but the proportion of the parasite mRNA may not be as great as the paired egg-producing adults.

Gene expression in schistosomula obtained *in vivo* (IVS) and *in vitro* (MTS)

Combined and filtered data from microarrays revealed differential gene expression of 6662 genes with a p value of ≤ 0.001. Of these sequences, 3207 were found to be over-expressed in MTS compared with IVS, and 3455 were found to be over-expressed in IVS compared with MTS, the distribution of relative gene expression is presented as a scatterplot in Fig. 3b. The percentage of major GO categories present within these differentially expressed genes is presented in Fig. 4. Genes over-expressed in MTS contained 348 (3208 individual GO categories) genes with an associate GO, while the 391 (3458 individual GO allocations categories) genes that were over-expressed in IVS had associated GO. Complete lists of the differentially expressed genes are presented in Supplementary Tables 3 and 4.

SGTPs, fatty acid transporters and enzymes involved in hemoglobin digestion are over-expressed in schistosomula produced *in vitro*. Contig8527 (SGTP1) and Contig4074 (SGTP4) were over-expressed 5.08-fold (p = 1.10E–38) and 4.75-fold (p = 1.72E–17), respectively, in MTS compared with IVS (Table 3), suggesting up regulation of these transcripts or SGTP-related sequences in the former. It is likely that the differential SGTP expression in the lung and *in vitro*-obtained schistosomula results from exposure to different environmental stimuli. It is well recognized that the presence of high concentrations of glucose affects the energy metabolism of schistosome cercariae and schistosomula. For example, incubation of cercariae in glucose and/or glucose-rich media significantly accelerates the process of membrane remodeling during *in vitro* cercarial transformation, as indicated by the rapid appearance of SGTP4 in the apical membrane of the newly formed heptalaminate membrane [26, 27].

Another example of a gene related to metabolism demonstrating differential expression is shown by a probe to a known FABP [25] that was unregulated in IVS compared to MTS (Contig1965 *S. japonicum* FABP mRNA), where it was down-regulated 1.71-fold (p = 8.62E–21). This is another example of a probably deficiency in the culturing conditions of MTS parasites.

Enzymes involved in hemoglobin digestion were over-expressed (ranging from ~2-to 13-fold) in MTS compared with IVS (Table 3). This is surprising, as *in vitro* culture medium contained serum, glucose, lactalbumin hydrolysate and vitamins, but not host erythrocytes. It is possible that the induction of hemoglobin-digesting enzymes in these schistosomula occurred in response to some nutritional or metabolic deficit of the culture media. If, as hypothesized above, IVS have a functional gut and actively ingest and digest host erythrocytes, schistosomula cultured in a medium lacking erythrocytes may be stimu-

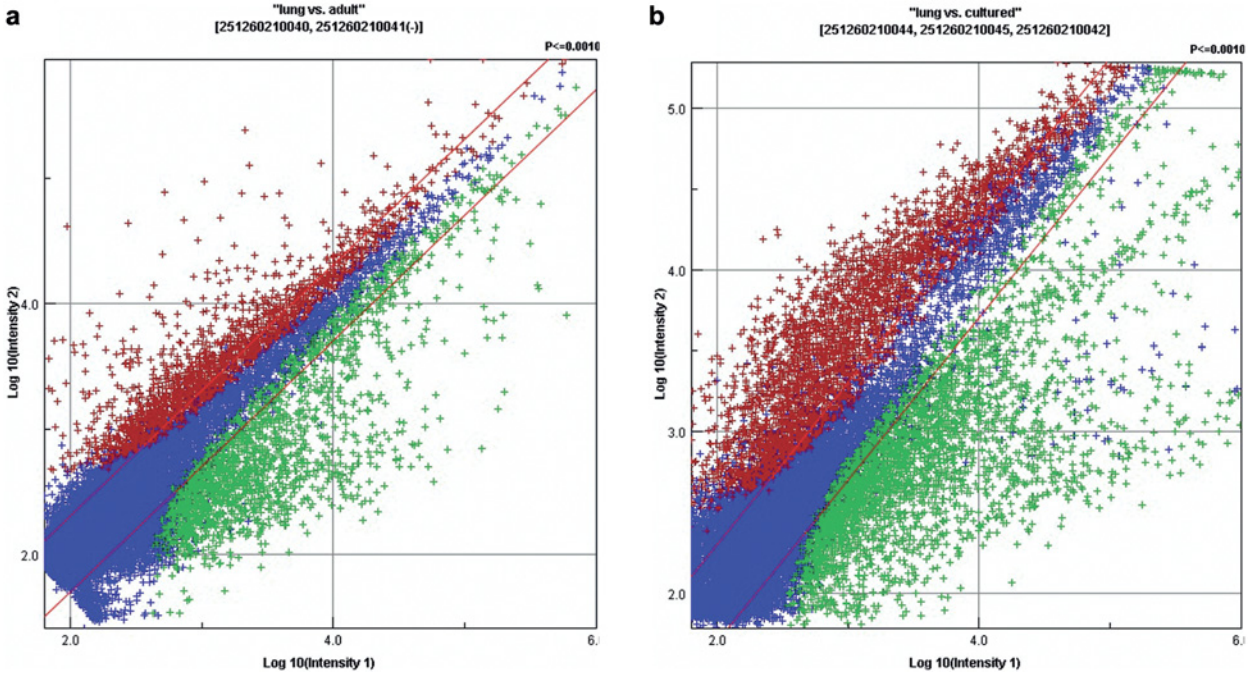

Figure 3. Scatterplots of log10 intensity (x) *versus* log10 ratio (y) for each oligonucleotide probe from one biological batch of one *S. japonicum* stage compared with another. (*a*) Comparison plot of microarray analysis of adult mixed-sex worms and *in vivo*-isolated lung schistosomula. Red, over-expression in adults; green, over-expression in lung schistosomula; blue, no differential expression detected below p = 0.001. (*b*) Comparison plot of microarray analysis of MTS and IVS. Red, over-expression in MTS; green, over-expression in IVS; blue, no differential expression detected below p = 0.001.

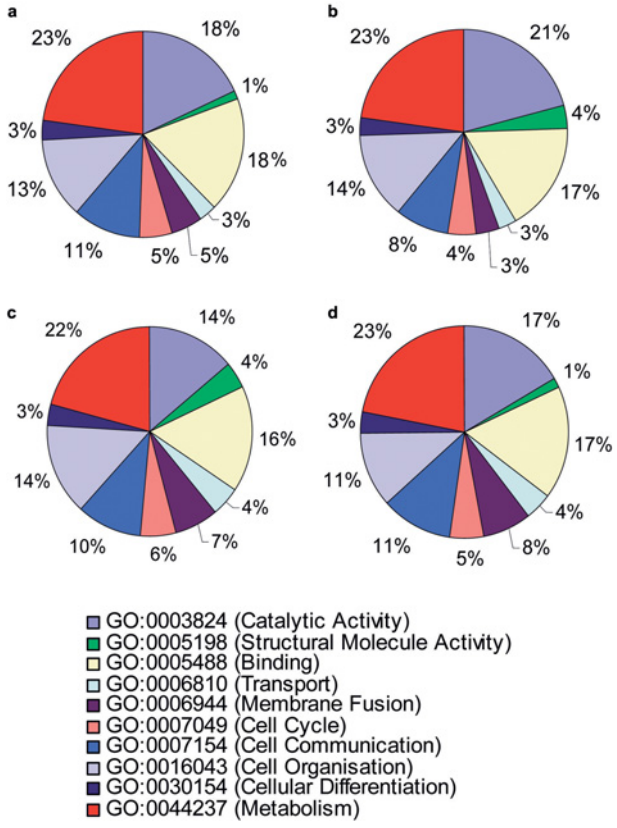

Figure 4. Percentage of gene ontology (GO) categories present for differentially expressed genes. Note individual probes may have multiple GO assignments. (*a*) Over-expressed probes in the MTS, compared to IVS. (*b*). Over-expressed genes in the adult parasite, compared to the lung schistosomula (IVS). (*c*) Over-expressed genes in IVS, compared to MTS. (*d*) Over-expressed genes in the lung schistosomula (IVS), compared to the adult parasite.

lated to over-express enzymes involved in hemoglobin digestion in an attempt to compensate for deficits in the culture medium.

Genes associated with immune evasion and stress responses are over-expressed in lung schistosomula

Anti-inflammatory, immunomodulatory protein Sm16. Anti-inflammatory responses include the closure of endothelial junctions, decreased trafficking of molecules and cells such as leukocytes, which reduces the expression of adhesion molecules such as E-selectin and vascular cell adhesion molecule 1 (VCAM-1) mRNA in human lung microvascular endothelial cells under inflammatory stimulation [28, 29]. *S. mansoni* cercariae secrete a potent anti-inflammatory protein, Sm16, which down-regulates the expression of interleukin-1α in human keratinocytes, decreasing keratinocyte motility. Sm16 also prevents lymphoproliferation and suppresses intracellular adhesion molecule-1 (ICAM-1) expression on endothelial cells [30, 31]. A probe designed to bind a *S. japonicum* assembled sequence homologous to Sm16 (AF269252) is present on the custom array, and the sequence was found to be over-expressed 8.58-fold ($p = 0$) in lung schistosomula compared with adults (Table 2). This supports the finding of Rao and Ramaswamy [30] who reported expression of Sm16 in sporocysts, cercariae, *in vitro*-transformed schistosomula and adults of *S. mansoni*, with highest expression in schistosomula, as determined by immuno-blot analysis of excreted/secreted protein products. These findings suggest an enhanced role of this im-

Table 3. Selected genes involved in nutritional metabolism up-regulated in mechanically transformed/*in vitro* cultured schistosomula of *S. japonicum*.

Gene	Contig	Fold*	p value
S. japonicum, homology to *S. mansoni* glucose transporter protein (SGTP1)	Contig8527	5.08	1.10E–38
S. japonicum, homology to *S. mansoni* glucose transporter protein (SGTP4)	Contig4074	4.75	1.72E–17
S. japonicum cathepsin B endopeptidase	Contig7921	12.78	1.36E–17
S. japonicum cathepsin B endopeptidase	Contig8839	3.64	1.91E–12
S. mansoni cathepsin C	Contig8499	2.88	3.09E–14
S. japonicum preprocathepsin L	Contig8250	2.20	2.25E–03
Candida albicans serine/threonine protein	Contig2944	3.39	1.75E–05
Euglena gracilis EPK2	Contig3917	6.70	5.01E–05
A. thaliana putative isocitrate dehydrogenase	Contig5418	8.45	0
S. mansoni tryptophan hydroxylase	Contig5573	2.03	7.01E–09
Xenopus laevis adenylosuccinate synthetase 2,	Contig7465	3.67	1.27E–09
Thellungiella halophila lipid transfer protein 4-	Contig8038	2.11	1.43E–04
Cathepsin B endopeptidase (*S. mansoni*)	TC10560	6.27	6.12E–38
Dolichyl-diphosphooligosaccharide-protein glycotransferase (*Branchiostoma belcheri tsingtaunese*)	TC10667	4.28	2.38E–20
Similar to GDP-L-fucose synthetase	TC8856	3.90	3.78E–04

* Fold changes are expressed as the ratio of gene expression in mechanically transformed schistosomula/*in vitro*-cultured schistosomula (Cy5)/lung schistosomula (Cy3).

Table 4. Some genes involved in immune evasion and the stress response up- or down-regulated in mechanically transformed/*in vitro*-cultured schistosomula of *S. japonicum.*

Gene	Contig	Fold*	p value
S. japonicum homologous to *S. mansoni* anti-inflammatory protein 16 mRNA (Sm16)	Contig7271	–3.99	1.59E–26
S. mansoni 28-kDa glutathione S-transferase	TC10486	–2.16	3.98E–09
Homology to *S. japonicum* muscle paramyosin (U11825) and *S. japonicum* paramyosin mRNA (D28811)	TC16824	–14.65	0
Homology to *S. japonicum* structural muscle paramyosin and *S. japonicum* mRNA sequence for paramyosin	Contig5188	–5.24	8.69E–21
Unknown *S. japonicum* clone with homology to *S. japonicum* structural muscle protein paramyosin	Contig4257	2.91	4.52E–27
S. japonicum clone (AY915153) unknown mRNA with homology to *S. mansoni* HSP70	Contig1898	–12.10	0
S. japonicum clone (AY813185) homology to *S. japonicum* P18 HSP70 (AF044413)	Contig8835	1.70	4.18E–11
S. mansoni immunophilin FK506 binding protein FKBP12	Contig1512	3.81	0
M. musculus Ig/EBP-1 gene for immunoglobulin enhancer binding protein	Contig4357	3.07	4.43E–04
Trichosurus vulpecula immunoglobulin kappa light chain variable region	Contig5116	5.32	5.90E–08
Homo sapiens immunoglobulin heavy locus (IGH.1@) on chromosome 14	Contig4964	–23.58	0

* Fold changes are expressed as the ratio of gene expression in mechanically transformed lung/*in vitro* cultured schistosomula (Cy5)/lung schistosomula (Cy3).

munomodulatory protein in the schistosomulum during passage through the lung when the parasite interacts with immunoreactive lung microvasculature endothelial cells. The Sm16 gene homologue was also found to be over-expressed 3.99-fold (p = 1.59E–26) in IVS compared with MTS (Table 4). This suggests that, as well as the developmental pattern of stage-specific Sm16 expression [30], Sm16 expression may additionally be regulated by environmental factors. It seems likely that exposure of lung schistosomula to lung microvascular endothelial cells and various immune factors (cytokines, IgE) and immune cells, such as mast cells, macrophages, eosinophils and basophils, may affect the expression of genes involved in immune evasion [32]. If so, it is likely that the expression of many genes relating to immune evasion may be depressed in MTS due to the lack of host-specific triggers or immune challenge.

Prostaglandins and 28-kDa glutathione-S-transferase. Prostaglandins (PG) are excretory/secretory products utilized by schistosomes to modulate the host immune system. The production of PGD_2 by *S. mansoni* mediates various regulatory functions of inflammation, and inhibits the migration of epidermal Langerhans cells (which play a key role in immune defense mechanisms) to the draining lymph nodes [33, 34]. The primary schistosome enzyme that synthesises PGD_2 is the 28-kDa glutathione-S-transferase (Sm28GST), excreted by skin-phase schistosomula [34, 35]. The Sm28GST gene (Contig 7271) showed no differential expression between adult and lung schistosomula. Sm28GST was, however, found to be over-expressed 2.16-fold (p = 3.98E–09) in IVS compared with MTS (Table 4), again indicating that host environmental factors can trigger the expression of a particular gene.

Paramyosin is over-expressed in lung schistosomula. Molecules that are associated with, or which are anchored to the tegument may also assist lung schistosomula in immune evasion. Paramyosin, a protein found in the muscle of adult schistosomes, cercariae and lung schistosomula, has also been identified on the tegumental surface of the lung parasite [36]. A surface-exposed non-filamentous form of paramyosin has been shown to inhibit the classical complement pathway by binding and inhibiting complement proteins [37]. Additionally, tegument expressed paramyosin also acts as a receptor for the Fc region of antibodies, possibly adsorbing antibodies onto the parasite surface [38, 39]. Multiple probes for clustered sequences or unknown clones bearing homology to *S. japonicum* paramyosin, and a probe for *S. mansoni* paramyosin are located on the custom array. The *S. mansoni* paramyosin sequence (GenBank acc. no. M35499) bears homology to two *S. japonicum* sequences, one for structural muscle protein paramyosin (acc. no. U11825), and one for a homologous paramyosin mRNA sequence (acc. no. D28811). The probe for the *S. mansoni* sequence (TC16824) was the only paramyosin probe with a significant p value in adults compared with lung schistosomula, and it was over-expressed 8.93-fold (p = 3.62E–37) in the latter (Table 2). This probe (TC16824) was also over-expressed 14.65-fold (p = 0) in IVS when compared to MTS (Table 4).

Another probe (Contig5188) for a *S. japonicum* assembled sequence homologous to the *S. japonicum* structural muscle paramyosin was over-expressed 5.24-fold (p = 8.69E–21) in IVS compared with MTS. It is possible that TC16824 and Contig5188 probes bind sequences for tegument expressed forms of paramyosin as they both represent paramyosin homologues that differ in sequence to the *S. japonicum* structural muscle protein. Another probe (Contig4257) for an unknown *S. japonicum* clone, homologous to *S. japonicum* structural muscle paramyosin, was over-expressed 2.91-fold (p = 4.52E–27) in MTS compared to IVS. These results support the view that paramyosin isoforms exist in schistosomes, and that the lung environment stimulates up-regulation of the putative tegument associated forms.

Stress proteins. Stress proteins are induced by exposure of an organism to stress factors such as heat, nutrient deprivation and metabolic disruption [40]. Such stresses would occur frequently during the schistosome life cycle, such as when cercariae emerge from the fresh-water snail host, enter an aquatic environment, penetrate the skin of the mammalian host and migrate via the lungs to the mesenteric veins [40, 41]. The HSP70 group in schistosomes is inducible by heat stress, and these proteins are transiently expressed at high levels during cercarial transformation and constitutively expressed in adult worms [42]. Various HSP70 genes are known, including *S. mansoni* HSP70 (acc. no. L02415) and *S. japonicum* P18 HSP70 mRNA (acc. no. AF044413) [40]. Using the custom array, an HSP70 probe (Contig6517) for an unknown *S. japonicum* clone (acc. no. AY810616) with sequence homology to *S. mansoni* HSP70 (acc. no. L02415) showed that this transcript was over-expressed 3.46-fold (p = 3.08E–25) in lung schistosomula compared to adult parasites (Table 2). Similarly, Neumann et al. [42] reported higher levels of HSP70 mRNA in 24-h mechanically transformed schistosomula than in adult *S. mansoni*, as indicated by Northern blot hybridization analysis for HSP70 sequences. Up-regulation of HSP70-related sequences in lung schistosomula may be associated with acquisition of heat tolerance, as the parasites have been in the host for only 3 days and might still be in a state for thermal flux [42], or perhaps tolerance to other multifactorial stresses imposed by, for example, host immune attack. A probe (Contig1898) for another *S. japonicum* clone, homologous to *S. mansoni* HSP70, was over-expressed 12.1-fold (p = 0) in IVS compared to MTS schistosomula (Table 3). This suggests that the stress stimulus for the over-expressed HSP is not uniquely related to heat shock as, following mechanical transformation, schistosomula were immediately subjected to *in vitro* culture at a temperature of 37 °C. It is likely that other stress stimuli provided by the mammalian lung environment, but not by *in vitro* culture, trigger up-regulation of stress proteins within *in vivo*-produced schistosomula. In like fashion, the opposite may also be true, because another sequence homologue of HSP (Contig8835) was over-expressed 1.7-fold (p = 4.18E–11) in MTS compared to IVS (Table 4), possibly in response to the lack of essential nutrients in the culture medium. In light of the fact that lung schistosomula may require erythrocytes or other blood proteins as nutrients, it is likely that their absence in culture media may result in the up-regulation of certain stress proteins, as has been hypothesized previously [40].

Conclusions

In summary, gene expression profiling of lung schistosomula and adult *S. japonicum* revealed differential expression of genes implicated in the host-parasite relationship. The genes that were differentially expressed are relevant to nutrient acquisition, immune evasion and the stress response, demonstrating adaptation to the different host environmental factors encountered by the two distinct schistosome life stages. These differentially expressed genes may also represent potential targets for vaccine or drug therapies.

The extensive differences in gene expression between schistosomula directly obtained from the lung compared with mechanically transformed/*in vitro*-cultured parasites indicate that the latter do not genetically represent *in vivo* parasites of the same age, resulting in under- or over-estimation of actual parasite gene expression. This is of major importance as the lung schistosomulum is a recognized target of vaccine-induced protective immunity [4]. A previous report has shown that *in vitro*-cultured schistosomula injected intravenously into a definitive host can survive [43]. It is, however, not apparent what rapid changes to gene expression are required during re-introduction into the host, and whether any of these changes occur to the parasite when in the lungs. The use of different experimental models as a definitive host may provide insight into this issue. The examination of early stage parasites from non-permissive hosts or primate hosts may provided further information on natural immunity to these parasites. Schistosomes obtained *in vitro* have been used for biochemical and molecular studies based on the premise that their development can be simulated independently of the host environment, and that observed morphological similarities confer overall equivalence of *in vitro*-produced parasites with those obtained *in vivo*. It is likely, however, that host environmental factors, which cannot be reliably reproduced *in vitro,* influence the growth, development and overall biology of schistosomes. Although useful in studies of gross morphology or for investigating some patterns of schistosome development, the use of *in vitro*-cultured schistosomes and me-

chanically transformed/cultured schistosomula in molecular or biochemical studies should be carefully considered.

URLs

Microarray data are available at **GEO (Gene Expression Omnibus)** http://www.ncbi.nlm.nih.gov/geo/. Supplementary materials as listed below are available for download at http://www.qimr.edu.au/research/labs/donm/cmls.

Supporting Information

Supplementary Tables 1	Over-expressed genes in the lung schistosomula compared to the adult parasite.
Supplementary Tables 2	Over-expressed genes in the adult parasite compared to the lung schistosomula.
Supplementary Tables 3	Over-expressed genes in the lung schistosomula (IVS) compared to the mechanically transformed schistosomula (MTS).
Supplementary Tables 4	Over-expressed genes in the mechanically transformed schistosomula (MTS) compared to the lung schistosomula (IVS).
Supplementary Tables 5	Full layout of the microarray.
Supplementary Materials and methods	

Acknowledgements. The authors would like to thank Mary Duke (QIMR) for the maintenance of the *S. japonicum* life cycle and for the provision of parasites for analysis. G.N.G. is a NHMRC (Australia) Howard Florey Fellow. The financial support of the Sandler Centre for Basic Parasitic Research and The Wellcome Trust and NHMRC (Australia) (International Collaborative Research Grants Scheme Award) is acknowledged.

1 Hu W., Brindley P. J., McManus D. P., Feng Z. and Han Z.-G. (2004) Schistosome transcriptomes: new insights into the parasite and schistosomiasis. Trends Mol. Med. **10:** 217–225
2 Capron A., Riveau G. J., Bartley P. B. and McManus D. P. (2002) Prospects for a schistosome vaccine. Curr. Drug Targets Immune Endocr. Metabol. Disord. **2:** 281–290
3 Fenwick A., Savioli L., Engels D., Bergquist N. R. and Todd M. H. (2003) Drugs for the control of parasitic diseases: current status and development in schistosomiasis. Trends Parasitol. **19:** 509–515
4 Ross A. G. P., Bartley P. B., Sleigh A. C., Olds G. R., Li Y. S., Williams G. M. et al. (2002) Current concepts—Schistosomiasis. N. Engl. J. Med. **346:** 1212–1220
5 Hu W., Yan Q., Shen D. K., Liu F., Zhu Z. D., Song H. D. et al. (2003) Evolutionary and biomedical implications of a *Schistosoma japonicum* complimentary DNA resource. Nat. Genet. **35:** 139–147
6 Verjovski-Almeida S., DeMarco R., Martins E. A. L., Guimaraes P. E. M., Ojopi E. P. B., Paquola A. C. M. et al. (2003) Transcriptome analysis of the acoelomate human parasite *Schistosoma mansoni*. Nat. Genet. **35:** 148–157
7 Gobert G. N., Moertel L. P. and McManus D. P. (2005) Microarrays: new tools to unravel parasite transcriptomes. Parasitology **131:** 439–448
8 Harrop R. and Wilson R. A. (1993) Protein synthesis and release by cultured schistosomula of *Schistosoma mansoni*. Parasitology **107:** 265–274
9 Brink L. H., McLAren D. J. and Smithers S. R. (1977) *Schistosoma mansoni*: a comparative study of artificially transformed schistosomula and schistosomula recovered after cercarial penetration of isolated skin. Parasitology **74:** 73–86
10 Cousin C. E., Stirewalt M. A. and Dorsey C. H. (1981) *Schistosoma mansoni*: ultrastructure of early transformation of skin- and shear-pressure-derived schistosomules. Exp. Parasitol. **51:** 341–365
11 Basch P. F. and Basch N. (1982) *Schistosoma mansoni*: scanning electron microscopy of schistosomula, adults and eggs grown *in vitro*. Parasitology **85:** 333–338
12 Bogitsh B. J. (1989) Observations on digestion in schistosomes or 'Blood and Guts'. Trans. Am. Microsc. Soc. **108:** 1–5
13 Harrop R., Coulson P. S. and Wilson R. A. (1999) Characterization, cloning and immunogenicity of antigens released by lung-stage larvae of *Schistosoma mansoni*. Parasitology **118:** 583–594
14 Knox D. P. (2004) Technological advances and genomics in metazoan parasites. Int. J. Parasitol. **34:** 139–152
15 Huggett J., Dheda K., Bustin S. and Zumla A. (2005) Real-time RT-PCR normalisation; strategies and considerations. Genes Immun. **6:** 279–284
16 Dorsey C. H., Cousin C. E., Lewis F. A. and Stirewalt M. A. (2002) Ultrastructure of the *Schistosoma mansoni* cercaria. Micron **33:** 279–323
17 Smyth J. D. and Halton D. W. (1983) Fine structure of the gut epithelium of *Schistosoma mansoni*. In: The Physiology of Trematodes.pp. 446, Cambridge Univeristy Press, Cambridge
18 Halton D. W. (1997) Nutritional adaptations to parasitism within the platyhelminthes. Int. J. Parasitol. **27:** 693–704
19 Senft A. W. (1976) Observations in the physiology of the gut of *Schistosoma mansoni*. In: Biochemistry of Parasites and Host-Parasite Relationships. pp. 335–342, Van den Bosch H. (ed.), Elsevier/North-Holland Biomedical Press, Oxford
20 Yuen T., Wurmbach E., Pfeffer R. L., Ebersole B. J. and Sealfon S. C. (2002) Accuracy and calibration of commercial oligonucleotide and custom cDNA microarrays. Nucleic Acids Res. **30:** e48
21 Brindley P. J., Kalinna B. H., Dalton J. P., Day S. R., Wong J. Y. M., Smythe M. L. et al. (1997) Proteolytic degradation of host hemoglobin by schistosomes. Mol. Biochem. Parasitol. **89:** 1–9
22 Caffrey C. R., McKerrow J. H., Salter J. P. and Sajid M. (2004) Blood 'n' guts: an update on schistosome digestive peptidases. Trends Parasitol. **20:** 241–248
23 Skelly P. J., Tielens A. G. M. and Shoemaker C. B. (1998) Glucose transport and metabolism mammalian-stage schistosomes. Parasitol. Today **14:** 402–406
24 Skelly P. J. and Shoemaker C. B. (1996) Rapid appearance and asymmetric distribution of glucose transporter SGTP4 at the apical surface of intramammalian-stage *Schistosoma mansoni*. Proc. Natl. Acad. Sci. USA **93:** 3642–3646
25 Gobert G. N., Stenzel D. J., Jones M. K. and McManus D. P. (1997) Immunolocalization of the fatty acid-binding protein Sj-FABPc within adult *Schistosoma japonicum*. Parasitology **115:** 33–39
26 Horemans A. M. C., Tielens A. and van den Bergh S. G. (1992) The reversible effect of glucose on the energy metabolism of *Schistosoma mansoni* cercariae and schistosomula. Mol. Biochem. Parasitol. **51:** 73–80
27 Skelly P. J. and Shoemaker C. B. (2000) Induction cues for tegument formation during the transformation of *Schistosoma mansoni* cercariae. Int. J. Parasitol. **30:** 625–631
28 Trottein F., Descamps L., Nutten S., Dehouck M.-P., Angeli V., Capron A. et al. (1999) *Schistosoma mansoni* activates host microvascular endothelial cells to acquire an anti-inflammatory phenotype. Infect. Immun. **67:** 3403–3409
29 Trottein F., Nutten S., Angeli V., Delerive P., Teissier E., Capron A. et al. (1999) *Schistosoma mansoni* schistosomula reduce E-

selectin and VCAM-1 expression in TNF-a-stimulated lung microvascular endothelial cells by interfering with the NF-κB pathway. Eur. J. Immunol. **29:** 3691–3701
30 Rao K. V. N. and Ramaswamy K. (2000) Cloning and expression of a gene encoding Sm16, an anti-inflammatory protein from *Schistosoma mansoni*. Mol. Biochem. Parasitol. **108:** 101–108
31 Ramaswamy K., Salafsky B., Potluri S., He Y. X., Li J. W. and Shibuya T. (1995) Secretion of an anti-inflammatory, immunomodulatory factor by schistosomulae of *Schistosoma mansoni*. J. Inflamm. **46:** 13–22
32 Maizels R. M., Balic A., Gomez-Escobar N., Nair M., Taylor M. D. and Allen J. E. (2004) Helminth parasites—masters of regulation. Immunol. Rev. **201:** 89–116
33 Angeli V., Faveeuw C., Roye O., Fontaine J., Teissier E., Capron A. et al. (2001) Role of the parasite-derived prostaglandin D-2 in the inhibition of epidermal Langerhans cell migration during schistosomsiasis infection. J. Exp. Med. **193:** 1135–1147
34 Herve M., Angeli V., Pinzar E., Wintjens R., Faveeuw C., Narumiya S. et al. (2003) Pivotal roles of the parasite PGD2 synthase and of the host D prostanoid receptor 1 in schistosome immune evasion. Eur. J. Immunol. **33:** 2764–2772
35 Mountford A. P. and Trottein F. (2004) Schistosomes in the skin: a balance between immune priming and regulation. Trends Parasitol. **20:** 221–226
36 Gobert G. N., Stenzel D. J., Jones M. K., Allen D. E. and McManus D. P. (1997) *Schistosoma japonicum*: immunolocalization of paramyosin during development. Parasitology **114:** 45–52
37 Deng J., Gold D., LoVerde P. T. and Fishelson Z. (2003) Inhibition of complement membrane attack complex by *Schistosoma mansoni* paramyosin. Infect. Immun. **71:** 6402–6410
38 Loukas A., Jones M. K., King L. T., Brindley P. J. and McManus D. P. (2001) Receptor for Fc on the surface of schistosomes. Infect. Immun. **69:** 3646–3651
39 Laclette J. P., Shoemaker C. B., Richter D., Arcos L., Pante N., Cohen C. et al. (1992) Paramyosin inhibits complement C1. J. Immunol. **148:** 124–128
40 Scott J. C. and McManus D. P. (1999) Identification of novel 70-kDa heat shock protein-encoding cDNAs from *Schistosoma japonicum*. Int. J. Parasitol. **29:** 437–444
41 Hedstrom R., Culpepper J., Harrison R. A., Agabian N. and Newport G. (1987) A major immunogen in *Schistosoma mansoni* infections is homologous to the heat-shock protein Hsp70. J. Exp. Med. **165:** 1430–1435
42 Neumann S., Ziv E., Lantner F. and Schechter I. (1993) Regulation of HSP70 gene expression during the life cycle of the parasite helminth *Schistosoma mansoni*. Eur. J. Biochem. **212:** 589–596
43 Pearce E. J., Basch P. F. and Sher A. (1986) Evidence that the reduced surface antigenicity of developing *Schistosoma mansoni* schistosomula is due to antigen shedding rather than host molecule acquisition. Parasite Immunol. **8:** 79–94
44 Hughes T. R., Mao M., Jones A. R., Burchard J., Marton M. J., Shannon K. W. et al. (2001) Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. Nat. Biotechnol. **19:** 342–347