Microarray Analysis of Gene Expression Profiles of *Schistosoma japonicum* Derived from Less-Susceptible Host Water Buffalo and Susceptible Host Goat

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**Abstract**

*Background:* Water buffalo and goats are natural hosts for *S. japonicum* in endemic areas of China. The susceptibility of these two hosts to schistosome infection is different, as water buffalo are less conducive to *S. japonicum* growth and development. To identify genes that may affect schistosome development and survival, we compared gene expression profiles of schistosomes derived from these two natural hosts using high-throughput microarray technology.

*Results:* The worm recovery rate was lower and the length and width of worms from water buffalo were smaller compared to those from goats following *S. japonicum* infection for 7 weeks. Besides obvious morphological difference between the schistosomes derived from the two hosts, differences were also observed by scanning and transmission electron microscopy. Microarray analysis showed differentially expressed gene patterns for parasites from the two hosts, which revealed that genes related to lipid and nucleotide metabolism, as well as protein folding, sorting, and degradation were upregulated, while others associated with signal transduction, endocrine function, development, immune function, endocytosis, and amino acid/carbohydrate/glycan metabolism were downregulated in schistosomes from water buffalo. KEGG pathway analysis deduced that the differentially expressed genes mainly involved lipid metabolism, the MAPK and ErbB signaling pathways, progestosterone-mediated oocyte maturation, dorso-ventral axis formation, reproduction, and endocytosis, etc.

*Conclusion:* The microarray gene analysis in schistosomes derived from water buffalo and goats provide a useful platform to disclose differences determining *S. japonicum* host compatibility to better understand the interplay between natural hosts and parasites, and identify schistosomae target genes associated with susceptibility to screen vaccine candidates.

**Introduction**

Schistosomiasis japonica is caused by the trematode *Schistosoma japonicum* and is one of the most prevalent zoonotic diseases in many Asian countries. In China, there are currently 365,770 human schistosomiasis cases, thus *S. japonicum* remains an important public health concern [1,2]. Despite more than a half century of control efforts, there is no effective means for the control of this disease and no substantial progress in the last century of control efforts, there is no effective means for the control of this disease and no substantial progress in the control of this disease and no substantial progress in the control of this disease and no substantial progress in the control of this disease and no substantial progress in the control of this disease and no substantial progress in the control of this disease and no substantial progress in the control of this disease and no substantial progress in *S. japonicum* vaccine development has been made, thus treatment is mainly dependent on the drug praziquantel to kill the adult worm within the host. There is a wide range of hosts for *S. japonicum* that include at least 46 mammalian species, including humans and a variety of domestic and wild animals, such as rats, rabbits, dogs, cats, horses, yellow cattle, goats, sheep, donkeys, and monkeys, among others [3]. Previous studies have revealed that susceptibility of different types of hosts is varied, as mice, goats, and yellow cattle are more sensitive than rats and water buffalo for *S. japonicum* (Chinese strain) infections [1].

In China, uncontrolled schistosomiasis endemic areas are mostly distributed in marshland/lake and mountainous regions [4–6] and epidemiological surveys have revealed that domestic animals play important roles in schistosomiasis transmission [7]. Water buffalo and goats are major domestic animals reared in endemic areas of China, especially water buffalo can spread more eggs into the environment than humans or other animal hosts and, thus they are considered as primary transmission sources of schistosomiasis in endemic areas [8–9]. He et al. [10] infected mice, rats, guinea pigs, rabbits, goats, sheep, pigs, water buffalo, yellow cattle, horses and 12 other kinds of animals with *S. japonicum* under the same conditions and observed the development of parasites in these hosts for up to 60 weeks. Their results showed
that the developmental rate of *S. japonicum* in these hosts was quite different, with the highest infection rate of 60.3% in goats, 43.6% in yellow cattle, and 1% in water buffalo and horses [10]. Water buffalo and goats act as major natural reservoir hosts for schistosomiasis in China although their susceptibility to schistosome infection is quite different, as goats are more susceptible to *S. japonicum* and result in higher rates of oviposition and sustain more severe pathological damage than water buffalo; however, the molecular basis of these differences remains unknown.

Several large-scale microarray analyses were recently performed using schistosomes to study gender-, stage-, and strain-specific gene expression of *S. japonicum* and *Schistosoma mansoni* [11–15]. The results of these studies suggested that gene and protein expression analysis in worms from different susceptible hosts can provide useful information to further elucidate the schistosome/host relationship. Recent studies in our laboratory revealed that schistosomes from a susceptible host (BALB/c mice), a less susceptible host (Wistar rat), and a non-permissive host (*Microtus fortis*, the reed vole) displayed different mRNA and protein expression profiles, and the gene expression analysis suggested that these three hosts may have different response mechanisms to schistosome infection [16–18]. These studies also indicated that the gene or protein expression profiles of schistosomes from natural hosts were different than those from laboratory animals. To better elucidate the susceptibility mechanism of schistosome in natural hosts and to identify molecules which might affect schistosome development, we infected water buffalo and goats with *S. japonicum* then analyzed and compared differences in gene expression profiles of the parasites obtained from the two hosts using microarray analysis. Our results will aid in screening vaccine candidates or new drug targets for the control of schistosomiasis in natural hosts dwelling within endemic areas.

**Materials and Methods**

**Ethics Statement**

All procedures were carried out in accordance with guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The animal study protocol was approved by the Animal Care and Use Committee of the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, People’s Republic of China.

**Animals and infection**

Male water buffalo and goats (n = 3 each), 15–18 months old, free of parasitic helminths and other infectious diseases were used for experimental infection. All animals were purchased from schistosome non-endemic areas with similar body weights for each host and were housed in covered pens, cared for by trained animal keepers, and fed hay and a commercial pelleted ration. *S. japonicum* (Chinese strain) cercariae were obtained from the snail maintenance room at Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Shanghai, China). Water buffalo were challenged percutaneously with 2000 cercariae of *S. japonicum* through the upper back using the cover glass method and goats were challenged percutaneously with 400 cercariae through the inguinal groove [19]. The cercariae were shed at 20–25°C before challenge to ensure maximum vitality.

**Sample collection and worm observation**

The animals were sacrificed 7 weeks postinfection and the parasites were perfused through the hepatic portal vein. All worms were detached manually, counted, and the length and width of each was measured by the same investigator. The worms were measured using a motorized microscope equipped with an auto camera ACT-2U (Nikon, Japan) and controlled by a Nikon image analysis software (NIS-Elements). Next, the worms were washed in phosphate-buffered saline (PBS) and then fixed in 2.5% glutaraldehyde phosphate buffer solution. Some of the worm samples were washed with PBS (pH 7.4) three times for 15–30 min each, fixed for 1.5 h with 1% osmic acid, washed three times as before, dehydrated with gradient alcohol (30%, 50%, 70%, 80%, 90%, 95%, and 100%), vacuum dried, spurted for 3 min using an ion sputtering instrument, and then observed via scanning electron microscopy (SEM) (Hitachi H-600; Hitachi Medical Corporation, Tokyo, Japan). The other samples were further dehydrated with acetone twice, embedded in an embedding medium, cut in ultrathin 70-nm sections and then observed via transmission electron microscopy (TEM) (Hitachi H-600; Hitachi Medical Corporation, Tokyo, Japan).

**RNA extraction and microarray analysis**

Worm samples were collected and stored in RNAlater RNA stabilization reagent (Ambion, Carlsbad, CA, USA). Total RNA was extracted from the parasites (10 pairs each animal) collected from water buffalo and goats using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and purified using the RNAeasy mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. The RNA integrity and quality was evaluated using the Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA).

The microarrays used to analyze the gene expression profiles in schistosomes from water buffalo and goat were constructed by Agilent Technologies, Inc. and included 13,821 contiguous sequences (contigs) plus proprietary positive and negative controls. Contigs were based on the nucleotide sequences from a recent *S. japonicum* database. Full details of this schistosome microarray design have been deposited in the Gene Expression Omnibus (GEO) database with the platform accession number GPL10987. Microarrays were printed in an 8×15 k feature format. A 200-ng aliquot of total RNA from each sample was converted into complementary RNA, labeled with the fluorophore cyanine 3-CTP (CY3c) and hybridized according to the manufacturer’s instructions (Agilent Technologies, Inc.; One-Color Microarray-Based Gene Expression Analysis). Samples were examined at wavelengths of A260 and A550 using the ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) to determine yield, concentration, amplification efficiency, and abundance of CY3c. Two technical replicates were performed for each sample and three independent biological replicates were designed for each host.

**Feature extraction and data analysis**

Microarrays were scanned using an Agilent Microarray Scanner (G2565BA) at a wavelength of 550 nm. Hybridized slides were scanned as tagged image files (TIFF) and processed with the Feature Extraction 9.5.3.1 Image Analysis program (Agilent Technologies, Inc.) to produce standardized data for statistical analysis. All slides were assessed for background evenness by viewing the TIFF image using Feature Extraction. Feature extracted data was analyzed using the GeneSpring GX statistical tool (version 7.3.1; Agilent Technologies, Inc.). Microarray data were normalized using a normalization scenario for ‘Agilent FE one-color,’ which included ‘Data Transformation: Set measurements less than 5.0 to 5.0,’ ‘Per Chip: Normalize to 50th percentile,’ and ‘Per Gene: Normalize to median.’

Data sets were further analyzed based on one-color experiments that have been published elsewhere [20]. The gProcessed Signal
values were determined by the GeneSpring GX statistical tool
using Feature Extraction software (Agilent, Inc.), including aspects
of signal/noise ratio, spot morphology, and homogeneity. The
GgProcessed Signal represents signals after localized background
subtraction and includes corrections for surface trends. Features
were deemed absent when the processed signal intensity was >2-
fold of the value of the processed signal error value. Features were
deemed marginal when the measured intensity was at a saturated
value or if there was a substantial amount of variation in the signal
intensity within the pixels of a particular feature. Features that
were neither absent nor marginal were deemed present. Data points
were included only if they were present or present/absent and probes
or contigs were retained if all data points were present or present/
absent. All microarray data were submitted to the Gene Expression
Omnibus public database under the accession number GSE24615.
All statistical analyses were performed using R statistical language
software (www.r-project.org/) and the q-value was estimated using
the false discovery rate (FDR) as a control [21–22]. Heatmap and
principal component analysis (PCA) were plotted using Java
Treeview software (Stanford University, Stanford, CA, USA) and
a multidimensional scaling algorithm [23].

Gene ontology and pathway pattern analysis
Further analysis was performed using Blast2Go Batch BlastX
software (6-frame translation protein homology; http://www.
blast2go.de) [24] to evaluate differences in annotation between two
groups of data. The analysis of gene ontology (GO) terms
associated to genes considered differentially expressed in Group
B (schistosomes from water buffalo) compared to Group G
(schistosomes from goats) was performed using the combined
graph function of the software. GO correlations with relative gene
expression values were made using ErmineJ software [25]. Kyoto
Encyclopedia of Genes and Genomes pathway patterns of
differentially expressed genes of interest were analyzed using the
SBC Analysis System (http://sas.ebioservice.com/).

Real-time polymerase chain reaction (PCR) validation
Real-time PCR was used to validate a subset of genes predicted
to be differentially expressed in the microarray experiment. All
gene-specific primers were designed using PRIMER3 software
(http://frodo.wi.mit.edu/primer3/input.htm). Purified RNA from
mixed parasite samples from each animal in each group was used
for reverse transcription in a final volume of 20 μL using the
PrimerScript RT kit with gDNA Eraser (Cat.# DRR047; Takara
Bio, Inc., Shiga, Japan). Products were amplified using the SYBR
Premix Ex Taq (Cat.# DRR041A; Takara Bio, Inc.) in an ABI
7500 Real-time System (Applied Biosystems) with the following
profile: 50°C for 2 min, 95°C for 30 s; 40 cycles of 95°C for 5 s
and 60°C for 34 s; 95°C for 15 s, and 60°C for 1 min. Each
reaction was performed using 20 μL of cDNA from the RT
reaction in a final volume of 50 μL. Expression levels of S.
japonicum nicotinamide adenine dinucleotide dehydrogenase (Gen-
Bank accession no.: AY812950) were used as endogenous controls
within each sample. Relative levels of gene expression were
calculated using the 2−ΔΔCT method [26]. The correlation of
microarray and qPCR analysis was performed by SPSS 16.0 [27].

Results and Discussion
Morphology of schistosomes derived from two natural hosts
The infected goats displayed more serious disease manifestations
than the water buffalo, including diarrhea, atrepsia, egg-
deposition, and pathological liver damage [28]. The worm
recovery rate in water buffalo was 2.9±1.05%, which was much
lower than that in goats (49.50±5.50%). Compared to schisto-
somites from a third natural host, yellow cattle, schistosomes from water buffalo presented growth and paring retardation [29]. In this study, schistosomes from water buffalo also showed growth retardation compared to parasites from goats (Figure 1A and B). Parasitic length and width from the two hosts were obviously different, as results showed that the female/male worm lengths from water buffalo and goats were \(8.86 \pm 1.86 \text{ mm} / 8.66 \pm 1.23 \text{ mm}\) and \(14.20 \pm 0.84 \text{ mm} / 9.40 \pm 0.55 \text{ mm}\), respectively, and the width of female/male worms from water buffalo and goats were \(202.98 \pm 12.33 \text{ mm} / 259.54 \pm 14.57 \text{ mm}\) and \(267.40 \pm 15.24 \text{ mm} / 377.05 \pm 15.97 \text{ mm}\), respectively (Figure 1C and D).

Internal structure analysis at the ultrastructural level by SEM and TEM showed differences between worms from the two natural hosts. Of male schistosomes from water buffalo, the oral and ventral suckers were crimpled, tension loosed, and the esthema mastoid processes in the integument were flattened and vacuolated, while the surface crest and sensory papillae in male worms from goats were affluent, compact, and well arranged (Figure 2). The female schistosomes from water buffalo had collapsed oral suckers on both sides, depressed ventral suckers, and poorly distinguished genital pores beneath the ventral suckers, while female worms from goats were affluent with more esthema mastoid processes in the oral suckers and obvious genital pores next to ventral suckers (Figure 3).

A comparison of male worms from water buffalo and goats using TEM showed that those from water buffalo had more vacuolar structures in the teguments, no internal microvilli, and the cytoplasmic organelles were dissolved (Figure 4). The female worms from water buffalo and goats had no obvious differences in the tegument, but there were more and longer internal microvilli in female worms from goats than from water buffalo (Figure 5).

Global gene expression profiles in schistosomes from water buffalo and goat

Three biological replicates of schistosomes from each host were evaluated and the correlation between biological replicates was 0.99 for each host. All data have been deposited in the Gene Expression Omnibus database maintained by the National Center for Biotechnology Information (GEO Series accession no.: GSE24615) (http://www.ncbi.nlm.nih.gov/geo/). We used 485 significant transcript expression values of schistosomes from the two natural hosts \((p<0.05\text{, fold change (FC)} \geq 2)\) for hierarchical clustering. Different profiles could be clearly identified among schistosomes from water buffalo (Group B) and goats (Group G). Two main clusters separated the schistosome genes among the two different hosts and the gene expression patterns of groups B and G.
were clustered (Figure 6I). To evaluate the overall data structure, we plotted the first two principal components of a PCA to capture the overall variance of the samples in two dimensions. This analysis clearly separated the data into two subgroups, which clustered the biological replicates together and separated the samples by the host from which the schistosomes were derived (Figure 6II).

For genes to be considered differentially expressed, a \( \geq 2 \) FC in gene expression was required with a t-test p-value <0.05. Compared to parasites from water buffalo, the distribution of the up- and down-regulated genes are shown in the scatter plot in Figure 6-III(222 up-regulated and 263 down-regulated). More differentially expressed genes were found in schistosomes between water buffalo and goats than between water buffalo and yellow cattle (485 vs. 69) because the members of the latter pair were more closely related to each other phylogenetically. Most differentially expressed schistosome genes between water buffalo and yellow cattle are also found in schistosomes between water buffalo and goats (46/69 vs. 46/485). A heatmap is shown in Figure 7 and a gene list is shown in Table S1 and S2 for the common differentially expressed genes. The majority of them were analyzed in detail in our previous report [30]. In this study, we identified several additional important differentially expressed genes between worms from water buffalo and goats.

Real-time PCR validation of microarray data

Subsets of genes with different expression levels and various biological functions in schistosomes from water buffalo and goats were selected for real-time PCR analysis to validate the microarray transcription results. The primer sequences and validation results are listed in Table 1. The real-time PCR results matched the microarray data very well, including the directionality and fold changes, a significant correlation of 0.817 was observed in our data (Spearman’s Rho, p<0.01, n = 9), thereby validating the microarray results.

GO functional distribution and pathway pattern analysis of differentially expressed genes in schistosomes from water buffalo and goats

The differentially expressed genes were mainly involved in biological regulation, developmental processes, growth, metabolic processes, cellular processes, and reproduction, (Figure 8A). Molecular functional analysis revealed that most of these molecules were involved in binding, catalytic activity, transporta-
tion, molecular transduction, transcription regulation, and enzymatic regulation (Figure 8B). Cellular component analysis showed that most of these molecules were components of the cell, cell parts, organelles, organelle parts, and envelope (Figure 8C). GO analysis and other bioinformatic techniques were further applied to predict/analyze possible functions of the identified bioactive molecules.

Up-regulated genes in schistosomes from water buffalo compared with those from goats

Compared with schistosomes from goats, many genes related to lipid metabolism (prostaglandin synthase 1 (PGS-1), acyltransferase-like family member 8 (ACL-8), acetylcholinesterase 1 (AChE-1), and degenerative spermatocyte homolog 1 (DEGS-1)), genetic information processing (DNA primase, nucleoside diphosphate kinase (NDK), trans-inducing factor, etc.), folding, sorting and degradation (26S proteasome, bromodomain and tryptophan-aspartic acid (WD) repeat domain containing 3 (BRWD3), and ribosomal RNA-processing protein 4 (RrP4)) were significantly up-

Figure 4. Comparisons of male worms derived from water buffalo and goats by transmission electron microscopy (TEM). (A, B) tegument, 2,000 × magnification; (C–F) subtegument and inner structures; (C, D) 3,000 × magnification; (E, F) 10,000 × magnification. R, ridge; P, pore; V, vacuoles; LM, longitudinal muscle; CM, outer circular muscle; MV, microvilli; L, lipid droplet; ERGG, endoplasmic reticulum glycogen granule. doi:10.1371/journal.pone.0070367.g004
regulated in schistosomes from the less-susceptible host water buffalo (Table 2).

**Lipid metabolism.** The genes associated with lipid metabolism were overexpressed in schistosomes from water buffalo compared to those from goats, but the differentially expressed genes varied among the hosts. The up-regulated genes in schistosomes from water buffalo included PGS-1, ACL-8, AChE-1, and DEGS-1. Prostaglandins are found in most tissues and organs, and synthesized by almost all nucleated cells from essential fatty acids as autocrine and paracrine lipid mediators. Prostaglandins ligate G-protein-coupled receptors, a sub-family of cell surface seven-transmembrane receptors. Ten prostaglandin receptors on various cell types were reported recently and the diversity of the receptors indicates that prostaglandins act on an array of cells and have a wide variety of effects, such as inducing constriction or dilation in vascular smooth muscle cells, aggregation or disaggregation of platelets, and controlling hormone regulation, cell growth, and other processes [31]. Acylation and
deacylation are the most common eukaryotic protein post-translational modifications. Acyltransferase functions as an acylation/deacylation catalytic protein and may have multiple functions in regulation of protein biological activity and gene expression.

The parasites derived from water buffalo were stunted and increased expression of nervous system- and transport-related genes when compared to those from yellow cattle [29]. In this study, the AChE-1 and DEGS-1 genes were up-regulated in worms from water buffalo compared to those from goats. AChE-1 is a serine protease that belongs to the carboxylesterase enzyme family and hydrolyzes the neurotransmitter acetylcholine at the synapses and can also hydrolyze butyrylthiocholine. AChE-1 is found mainly at neuromuscular junctions and cholinergic brain synapses where it serves to terminate synaptic transmission. Although AChE-1 is detected at all growth stages of Caenorhabditis elegans, it is more abundant in larval stages than in embryos or adults [32]. DEGS-1 encodes a membrane-bound fatty acid desaturase, which is responsible for forming double bonds into specific positions in fatty acid molecules. DEGS-1 can up-regulate cyclin D1 expression and activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells [33]. Cyclin D1 plays a pivotal role in cell-cycle transition through the G1 phase and regulates cyclin-dependent kinase 6, which, in turn, regulates schistosome embryogenesis and worm paring through the transforming growth factor beta signaling pathway [34,35]. DEGS1 overexpression inhibited biosynthesis of the epidermal growth factor receptor (EGFR) [36] and in this study, the EGFR gene was down-regulated (CUST_3717, FC -4.52) in schisto-

Figure 6. Transcription profile analysis of significantly differentiated expressed genes in S. japonicum from water buffalo (group B) and goats (group G). (A) Hierarchical clustering using differentially expressed genes (probe sets) (p<0.05; FC>2); (B) Principal component analysis (PCA) of transcript profiles from groups B and G; (C) A scatter plot comparing groups G and B. The number at the upper left denotes up-regulated genes and the number at the lower right denotes down-regulated genes (p<0.05; FC>2).

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somes from water buffalo, which might be attributable to the DEGS1 over-expression.

Genetic information processing. Biological phenotypes were determined according to gene expression. The genetic information processing-associated genes showed significant differential expression in schistosomes among the hosts. Schistosomes cannot synthesize purines alone, thus they are dependent on a supply from the host [37]. Our results determined that purine and pyrimidine metabolism-associated genes were overexpressed in schistosomes from water buffalo compared to those from goats, including DNA primase (CUST_3246) and nucleoside-diphosphate kinase (CUST_9849). In addition to its catalytic activity of phosphorylation, NDK reportedly regulates growth and development [38] and is considered a housekeeping enzyme for DNA and RNA synthesis. However, NDK, as well as other transcription-, splicesome-, and translation-associated genes were upregulated in the less susceptible host water buffalo, suggesting that they are important for parasite retardation (Table 2).

Folding, Sorting, and Degradation. The differentially expressed molecules found in this class included posttranslational

| Probe name | Protein homology | B_C | B_G |
|------------|-----------------|-----|-----|
| CUST_11761 | homeobox protein distal-less like | -2.22 | -5.82 |
| CUST_4819  | tauine transporter | -2.07 | -2.13 |
| CUST_8465  | leucine-rich repeat protein scabrous complex protein | -2.70 | -7.43 |

Figure 7. Heatmap clustering of common differentially expressed genes. The right tables listed the common differentially expressed genes (p<0.05; FC>2). Schistosomes from water buffalo, yellow cattle, and goats were simplified as groups B, C, and G respectively. "-" indicates downregulation. FC is represented as the mean value. The tables do not contain genes without protein homology descriptions.

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modification molecules, as 26S proteasome (CUST_3409), BRWD3 (CUST_9175), and RrP4 (CUST_9175) were upregulated in schistosomes from water buffalo. The 26S proteasome is at the executive end of the ubiquitin proteasome pathway (UPP) for the controlled degradation of intracellular proteins. In eukaryotes, the UPP is essential for proteostasis, in which misfolded or otherwise defective proteins as well as short-lived regulatory proteins are eliminated by degradation [39]. The UPP regulates many fundamental cellular processes, such as protein quality control, DNA repair, cell cycle regulation, antigen processing, and signal transduction [40]. Lysine acetylation is similar to protein phosphorylation in its prevalence in posttranslational modifications and also has a large effect on the physicochemical properties of the modified residues. Protein recruitment to macromolecular complexes by acetylated lysine residues is mediated by bromodomains (BRDs), which are evolutionarily highly conserved protein interaction modules that recognize e-N-lysine acetylation motifs [41]. The WD repeat-N-lysine acetylation motifs provide a large number of evolutionarily both in prokaryotic and eukaryotic organisms [42]. Members of this family are involved in a variety of cellular processes, including cell cycle progression, signal transduction, apoptosis, and gene regulation [42]; however, little is known regarding the biological function of BRWD3. In Drosophila, BRWD3 function has been genetically linked to the Janus kinase-signal transducer and activator of transcription pathway [43]. Mutations in mice revealed a role for BRWD1 in spermiogenesis and oocyte–embryo transition [44]. RNA-binding proteins (RBPs) bind to either double-strand or single-strand RNA molecules through RNA recognition motifs. Thus, RBPs may regulate RNA translation and post-transcriptional events, such as RNA splicing and editing.

### Down-regulated genes in schistosomes from water buffalo compared with those from goats

Compared with schistosomes from goats, many genes related to signal transduction (i.e., Akt, ribosomal s6-p90 kinase (RSKN-1), mitogen-activated protein kinase kinase 1 (MEK-1), MEK-6, protein kinase-18 (KIN-18), EGFR, 70-kDa heat shock protein (HSP70), dishevelled (Dsh), etc.), endocrine function (Akt, RSKN-1, MEK-1), development (MEK-1, EGFR), reproduction (SBP), immune function (MEK-1), endocytosis (MEK-1, HSP70, EGFR, partitioning-defective 3 homolog (PAR-3), etc.), amino acid metabolism (aspertate aminotransferase, dopamine beta-hydroxylase, and aromatic-L-amino-acid decarboxylase), carbohydrate metabolism (PI3K-C2, PI4Kz, pyruvate carboxylase, and galactokinase 2 (GALK2)), and glycan biosynthesis, and metabolism (glycosylationdefective 3 homolog (GALD)) were significantly downregulated in schistosomes from the less-susceptible host water buffalo (Table 3).

### Signal transduction/Endocrine system/Development/Reproduction

Mitogen-activated protein kinase (MAPKs) are intracellular serine/threonine protein kinases. The study confirmed that the MAPK signal transduction pathway was present in most cells and transduction of extracellular signals to the cell and its nucleus played a crucial role in cellular biological responses, such as cell proliferation, differentiation, transformation, and apoptosis. MAPK signal transduction pathway was highly conserved evolutionarily both in prokaryotic and eukaryotic cells. The serine-threonine kinase Akt, also known as protein kinase B (PKB), is an important effector for phosphorylating insulin signaling initiated by numerous growth factors and hormones, and has also been implicated in many metabolic functions, such as protein and lipid synthesis, carbohydrate metabolism, and transcription [45]. The serine-threonine kinase Akt represents an important mediator of insulin action in worms and flies. In *C. elegans*, mutations in Akt result in alterations in growth and longevity [46,47]. Other studies have reported that Akt1−/− mice displayed a conspicuous growth impairment and demonstrated defects in both fetal and postnatal growth, which persisted into adulthood [48]. Akt1−/− mice were smaller when compared to wild-type littermates. In addition, Akt1−/− mouse embryo fibroblasts are more susceptible to

#### Table 1. Details of real-time RT-PCR primers and results of confirmation.

| ProbeName | Primer(5'-3') | Size (bp) | Real-time FC* | Microarray FC† | Description |
|-----------|--------------|----------|--------------|----------------|-------------|
| CUST_551  | FP:agaatgcggtgctaaacaa<br>Rp:atctgcccaggtataggaacaa | 196 | −2.63 | −6.84 | Expressed protein |
| CUST_12936 | FP:gaagctgtggtctgggtgc<br>Rp:atctgcggatcctcagcg | 192 | −2.64 | −7.27 | Prostatic spermine-binding protein precursor (SBP) |
| CUST_4819 | FP:aatagcgctgtaaaaggaag<br>Rp:acacacagctgtaaattgc | 210 | −2.05 | −2.14 | Solute carrier family 6 (ko:K05045) |
| CUST_12988 | FP:tgcttgtgqagggaggttt<br>Rp:tgaggtcaggcttgagcg | 219 | 50 | 12.56 | Putative uncharacterized protein C14orf165 |
| CUST_4112 | FR:gttattggatttcccgctca<br>PR:atggcgaataggggcatca | 199 | 3.34 | 6.34 | Cyclin-dependent kinase 6, CDK6 |
| CUST_10350 | FR:actctggatcttggtttgtag<br>PR:atctggatcttggtttgtag | 198 | 4.75 | 6.88 | Asparagine-rich protein (Ag319) (ARP) |
| CUST_10723 | FR:tgatcgcgttggtttgtag<br>PR:atgtgcccgggttggtttgtag | 178 | 2.11 | 4.73 | Expressed protein |
| CUST_4101 | FR:atctgccggatatgaaccaa<br>PR:agatcgcgttcaaacaacaa | 230 | 3.52 | 4.88 | Iroquois homeobox protein 3, IRX-3 |
| CUST_5523 | FR:gcggtgctcaaatgtatgaatt<br>PR:gtgtgtgctcagggggtt | 184 | 5.2 | 2.56 | Putative eukaryotic translation initiation factor 3 subunit (eIF-3) |

*Fold change (FC) is the ratio of gene expression in schistosomes from water buffalo compared to those from goats; p<0.05, FDR<0.1; †Mean FC in real-time PCR results for validation.

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**Figure 1.** Gene expression in schistosomes from different hosts. The 26S proteasome is at the executive end of the ubiquitin proteasome pathway (UPP) for the controlled degradation of intracellular proteins. In eukaryotes, the UPP is essential for proteostasis, in which misfolded or otherwise defective proteins as well as short-lived regulatory proteins are eliminated by degradation [39]. The UPP regulates many fundamental cellular processes, such as protein quality control, DNA repair, cell cycle regulation, antigen processing, and signal transduction [40]. Lysine acetylation is similar to protein phosphorylation in its prevalence in posttranslational modifications and also has a large effect on the physicochemical properties of the modified residues. Protein recruitment to macromolecular complexes by acetylated lysine residues is mediated by bromodomains (BRDs), which are evolutionarily highly conserved protein interaction modules that recognize e-N-lysine acetylation motifs [41]. The WD repeat-N-lysine acetylation motifs provide a large number of evolutionary conserved motifs in prokaryotic and eukaryotic organisms [42]. Members of this family are involved in a variety of cellular processes, including cell cycle progression, signal transduction, apoptosis, and gene regulation [42]; however, little is known regarding the biological function of BRWD3. In Drosophila, BRWD3 function has been genetically linked to the Janus kinase-signal transducer and activator of transcription pathway [43]. Mutations in mice revealed a role for BRWD1 in spermiogenesis and oocyte–embryo transition [44]. RNA-binding proteins (RBPs) bind to either double-strand or single-strand RNA molecules through RNA recognition motifs. Thus, RBPs may regulate RNA translation and post-transcriptional events, such as RNA splicing and editing.
apoptosis induced by the tumor necrosis factor receptor superfamily, UV irradiation, and serum withdrawal [49]. Nonetheless, much uncertainty remains concerning how this signaling pathway diverges to allow independent regulation of such disparate biological processes as metabolism, aging, and growth. In schistosomes from water buffalo, the Akt gene was significantly down-regulated (CUST_11642, FC \(2.69\)), suggesting that Akt may be involved in growth retardation via MAPK signal transduction or the endocrine system.

The RSKN-1 protein is a downstream effector of MPK-1/ERK and is critical for dedifferentiation. Rskn-1 RNAi suppressed spermatocyte dedifferentiation and instead induced meiotic division in the \(C. elegans\) germline. These regulators are broadly conserved, suggesting that similar molecular circuitry may control cellular dedifferentiation in other organisms as well [50]. The Rskn-1 gene was underexpressed in schistosomes from water buffalo (CUST_12915, FC \(2.48\)) and might also be associated with cellular dedifferentiation; however, this hypothesis requires further investigations.

KIN-18 encodes a previously uncharacterized protein in \(C. elegans\) and its catalytic domain shares over 60% identities with TAO kinase 1 (TAO1) and TAO2, which have been recently characterized with a MAPK/ERK kinase role during stress response. Berman et al. [51] reported that expression of constitutively active forms of TAO1/KIN-18 affect the physiology of intact worms and demonstrated that KIN-18 was a 120-kDa protein and its promoter was active in the pharynx and intestine of \(C. elegans\). These worms grow more slowly, lay fewer eggs, and phenotypes could result from reduced feeding. Other research has suggested a role for MEK-1 in stress responses, with a focus in the pharynx and/or intestine [52]. The KIN-18 (CUST_9047, \(15.09\)) and MEK-1 (CUST_3675, \(4.15\)) genes were significantly downregulated in schistosomes from water buffalo, which may be attributed to the morphological defect in worms from water buffalo compared to those from goats, as reduced nutritional intake can retard worm growth.

EGFR belongs to the ErbB family of receptor tyrosine kinases, which possess protein tyrosine kinase activity and are found only in metazoans [53]. The downstream signaling proteins initiate several signal transduction cascades, principally the MAPK, Akt, and mitogen-activated protein kinase 8 isoform beta 2 pathways, leading to DNA synthesis and cell proliferation. EGFR plays an important role in development and cell differentiation, and EGFR homologues have been identified in a broad range of vertebrate and invertebrate organisms [54]. Here, we found that EGFR was differentially underexpressed in schistosomes from water buffalo.
EGFR can act as a target regulated by host factors, resulting in different worm compatibility within different hosts, thereby supporting the hypothesis that host EGF can regulate *S. mansoni* development [55].

Hsp70 proteins are ubiquitously expressed and exist in virtually all living organisms. The Hsp70 proteins are an important part of the cellular machinery for protein folding and help to protect cells from thermal or oxidative stress [56]. In addition to improving overall protein integrity, Hsp70 directly inhibits apoptosis by blocking the recruitment of procaspase-9 to the Apaf-1/dATP/cytochrome c apoptosome complex [57]. Given that water buffalo is the less suitable host, the Hsp70 gene of schistosomes from water buffalo was down-regulated (CUST_5301 FC, -2.24), therefore Hsp70 is predicted to promote parasitic apoptosis in vivo [58].

Dsh is a family of proteins involved in canonical and non-canonical integration/ wingless (Wnt) signaling pathways. Dsh is a cytoplasmic phosphoprotein that acts directly downstream of frizzled receptors [59] and plays important roles in both the embryo and the adult, ranging from cellular differentiation and cell polarity to social behavior [60]. However, the role of Dsh down-regulation in schistosomes from water buffalo (CUST_5485, -4.48) has not yet been investigated.

Prostatic spermine-binding protein (SBP) binds to forkhead protein A1 (FoxA1) and FoxA1 is critical for the androgenic regulation of prostate-specific promoters. Androgen plays a key role in the normal prostate development and physiology [61]. Female worms require stimulation from male worms to achieve and maintain a mature reproductive state. The SBP gene was significantly underexpressed in schistosomes from water buffalo (CUST_12936, 27.27), which was predicted to have a role in reproduction processes by influencing interactions among male and female worms, resulting in parasitic retardation and less egg deposition in the liver [28].

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### Table 2. Selected genes overexpressed in schistosomes from water buffalo compared to those from goats.

| Pathway                                      | Probe name | Accession | Gene and/or protein homology | Fold change | p value | FDR     |
|----------------------------------------------|------------|-----------|------------------------------|-------------|---------|---------|
| Lipid Metabolism                             | CUST_3764  | CNUS0000098059 | Prostaglandin synthase 1 | 2.19        | 0.021   | 0.007   |
|                                              | CUST_7726  | CNUS0000102024 | Acetylcholinesterase 1     | 2.71        | 0.036   | 0.007   |
| Glycerophospholipid metabolism & Glycerolipid metabolism | CUST_8880  | CNUS0000103178 | Acyltransferase-like family member 8 | 2.57 | 0.026 | 0.008 |
| Sphingolipid metabolism                      | CUST_6882  | CNUS0000101178 | Degenerative spermatocyte homolog 1 | 2.45 | 0.021 | 0.008 |
| Purine metabolism, Pyrimidine metabolism     | CUST_3246  | CNUS0000097541 | DNA primase small subunit    | 2.02        | 0.023   | 0.01    |
|                                              | CUST_9849  | CNUS0000104147 | Nucleoside-diphosphate kinase | 2.08        | 0.001   | 0       |
| Transcription                                | CUST_9094  | CNUS0000103392 | Transcription initiation factor TFIIE alpha | 2.34 | 0.018 | 0.008 |
| Spliceosome                                  | CUST_11072 | CNUS0000105370 | Hypothetical protein        | 2.62        | 0.036   | 0.01    |
|                                              | CUST_488   | FN317168   | SJFC0697                    | 3.69        | 0.022   | 0.008   |
|                                              | CUST_7382  | CNUS0000101679 | Expressed protein            | 4.53        | 0.019   | 0.007   |
| Translation                                  | CUST_6052  | CNUS0000100348 | Small subunit ribosomal protein S27Ae | 4.71 | 0.002 | 0.007 |
| Folding, Sorting and Degradation             | CUST_3409  | CNUS0000097704 | 26S proteasome regulatory subunit N12 | 3.26 | 0.001 | 0.006 |
| Proteasome                                   | CUST_9175  | CNUS0000103473 | Bromodomain and WD repeat domain containing 3 | 2.55 | 0.001 | 0.006 |
| Ubiquitin mediated proteolysis               | CUST_2890  | CNUS0000097185 | RNA-binding protein Rlp4 and related proteins | 6.13 | 0.020 | 0.007 |

*This list includes the probe name, gene accession number, fold change, as well as p- and q-values for protein homology (BlastX). In the gene accession columns, data with identifiers such as CNUS0000098407 were retrieved from the *S. japonicum* genome project database (LSBI; http://lifecenter.sgst.cn/schistosoma/cn/genomeProject.do; in Japanese) and those with identifiers FN326902 were retrieved from The European Molecular Biology Laboratory database (http://www.ebi.ac.uk/ena/). The FDR (q-value) was determined using the false discovery rate as a control using R statistical language (http://www.bioconductor.org/packages/release/bioc/html/qvalue.html). doi:10.1371/journal.pone.0070367.t002*
### Table 3. Selected downregulated genes in schistosomes from water buffalo compared with those from goats.a

| Pathway                              | Probe name                     | Accession          | Gene and/or protein homology                                      | Fold change | p value   | FDR   |
|--------------------------------------|--------------------------------|-------------------|------------------------------------------------------------------|-------------|-----------|-------|
| **Signal transduction**              |                                |                   |                                                                  |             |           |       |
| MAPK signaling pathway               | CUST_11642                     | CNUS0000010594    | Akt1;RAC serine/threonine-protein kinase                         | −6.92       | 0.022     | 0.005 |
|                                      | CUST_12915                     | CNUS00000107219   | Protein RSKN-1                                                  | −2.48       | 0.018     | 0.005 |
|                                      | CUST_3675                      | CNUS00000097970   | MAPK/ERK kinase 1(MEK-1)                                        | −4.15       | 0.0002    | 0     |
|                                      | CUST_9010                      | CNUS00000103308   | MAPK/ERK kinase 6(MEK-6)                                        | −2.91       | 0.003     | 0     |
|                                      | CUST_9047                      | CNUS00000103345   | Protein KIN-1                                                   | −15.09      | 0.0003    | 0     |
|                                      | CUST_3717                      | CNUS00000098012   | Epidermal growth factor receptor                                | −4.52       | 0.004     | 0.005 |
|                                      | CUST_9487                      | CNUS00000103785   | Probable beta-arrestin                                          | −2.33       | 0.040     | 0.007 |
|                                      | CUST_5301                      | CNUS00000099597   | Heat shock 70 kDa protein cognate 4                             | −2.24       | 0.024     | 0.01  |
| ErB signaling pathway                | CUST_11642                     | CNUS00000105941   | Akt1;RAC serine/threonine-protein kinase                         | −6.92       | 0.022     | 0.005 |
|                                      | CUST_3675                      | CNUS00000097970   | MAPK/ERK kinase 1                                               | −4.15       | 0.0002    | 0     |
| Wnt signaling pathway, Notch signaling pathway | CUST_5485                     | CNUS00000099781   | Dishevelled protein(Dsh)                                        | −4.48       | 0.001     | 0.005 |
| **Endocrine System**                 |                                |                   |                                                                  |             |           |       |
| Progesterone-mediated oocyte maturation | CUST_11642                     | CNUS00000105941   | Akt1;RAC serine/threonine-protein kinase                         | −6.92       | 0.022     | 0.005 |
|                                      | CUST_12915                     | CNUS00000107219   | Protein RSKN-1                                                  | −2.48       | 0.018     | 0.005 |
| Development/Reproduction              | CUST_3675                      | CNUS00000097970   | MAPK/ERK kinase 1                                               | −4.15       | 0.0002    | 0     |
| Dorso-ventral axis formation         | CUST_3717                      | CNUS00000098012   | Epidermal growth factor receptor                                | −4.52       | 0.004     | 0.005 |
| Reproduction                         | CUST_12936                     | CNUS00000107240   | Prostatic spermine-binding protein (SBP)                        | −7.27       | 0.012     | 0.005 |
| **Immune System**                    |                                |                   |                                                                  |             |           |       |
| Natural killer cell mediated cytotoxicity | CUST_3675                     | CNUS00000097970   | MAPK/ERK kinase 1                                               | −4.15       | 0.0002    | 0     |
| **Endocytosis**                      |                                |                   |                                                                  |             |           |       |
|                                      | CUST_10516                     | CNUS00000104814   | beta-adrenergic-receptor kinase                                  | −2.05       | 0.0033    | 0     |
|                                      | CUST_3717                      | CNUS00000098012   | Epidermal growth factor receptor                                | −4.52       | 0.004     | 0.005 |
| **Amino Acid Metabolism**            |                                |                   |                                                                  |             |           |       |
|                                      | CUST_3675                      | CNUS00000097970   | MAPK/ERK kinase 1                                               | −4.15       | 0.0002    | 0     |
| **Carbohydrate Metabolism**          |                                |                   |                                                                  |             |           |       |
| Inositol phosphate metabolism        | CUST_10516                     | CNUS00000104840   | Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing alpha (PI3K-C2z) | −2.43       | 0.025     | 0.008 |
|                                      | CUST_13086                     | CNUS00000107391   | Phosphatidylinositol 4-kinase alpha (PI4Kα)                     | −2.03       | 0.027     | 0.01  |
| Citrate cycle (TCA cycle), Pyruvate metabolism | CUST_6418                     | CNUS00000100714   | Pyruvate carboxylase                                            | −2.61       | 0.002     | 0.005 |
| **Glycan Biosynthesis and Metabolism** |                                |                   |                                                                  |             |           |       |
| Glycosylphosphatidylinositol(GPI)-anchor biosynthesis | CUST_7443                     | CNUS00000101740   | Galactokinase 2(GALK2)                                          | −3.99       | 0.019     | 0.008 |
| N-Glycan biosynthesis                | CUST_9370                      | CNUS00000103668   | Glycoproteins(Protein GLY-2)                                    | −2.00       | 0.007     | 0.006 |

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Comparison of Schistosomes from Different Hosts

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thesis/metabolism-associated genes were accordingly downregulated.

A comparison of schistosomes from the less susceptible host (water buffalo) with those from the more susceptible host (goats) at the phenotype and gene expression levels. Except the length and width difference, SEM and TEM showed that the ultrastructure of worms from different hosts were very different. A total of 485 differentially expressed genes were identified and their gene expression patterns in schistosomes from water buffalo and goat hosts were identified here. Our results revealed that, compared with schistosomes from goats, genes involved in lipid metabolism, genetic information processing, folding, sorting and degradation were upregulated in schistosomes from water buffalo, whereas other genes associated with signal transduction, endocrine function, development, reproduction, endocytosis, amino acid/carbohydrate metabolism, immune function, etc. were downregulated. These events were deduced to be key differences for the survival and development of schistosomes in different compatible natural host environments.

The microarray analysis of gene expression differences in schistosomes derived from water buffalo and goat provided a useful platform to discover the differences in host compatibilities, and furthered our understanding of the interplay between natural hosts and parasites, and identified several schistosome target genes associated with susceptibility for the screening of vaccine candidates.

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