Comparative Study of Transcriptome Profiles of Mechanical- and Skin-Transformed *Schistosoma mansoni* Schistosomula

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Abstract

Schistosomiasis infection begins with the penetration of cercariae through healthy unbroken host skin. This process leads to the transformation of the free-living larvae into obligate parasites called schistosomula. This irreversible transformation, which occurs in as little as two hours, involves casting the cercaria tail and complete remodelling of the surface membrane. At this stage, parasites are vulnerable to host immune attack and oxidative stress. Consequently, the mechanisms by which the parasite recognises and swiftly adapts to the human host are still the subject of many studies, especially in the context of development of intervention strategies against schistosomiasis infection. Because obtaining enough material from in vivo infections is not always feasible for such studies, the transformation process is often mimicked in the laboratory by application of shear pressure to a cercarial sample resulting in mechanically transformed (MT) schistosomula. These parasites share remarkable morphological and biochemical similarity to the naturally transformed counterparts and have been considered a good proxy for parasites undergoing natural infection. Relying on this equivalency, MT schistosomula have been used almost exclusively in high-throughput studies of gene expression, identification of drug targets and identification of effective drugs against schistosomes. However, the transcriptional equivalency between skin-transformed (ST) and MT schistosomula has never been proven. In our approach to compare these two types of schistosomula preparations and to explore differences in gene expression triggered by the presence of a skin barrier, we performed RNA-seq transcriptome profiling of ST and MT schistosomula at 24 hours post transformation. We report that these two very distinct schistosomula preparations differ only in the expression of 38 genes (out of ∼11,000), providing convincing evidence to resolve the skin vs. mechanical long-lasting controversy.

Introduction

Schistosomiasis is a parasitic disease caused by platyhelminths of the genus *Schistosoma*. It has been estimated that ∼200 million people are infected and ∼200,000 die due to schistosomiasis-related pathologies [1]. Without a vaccine, mechanisms of prophylaxis rely primarily on reduction of the number of infected individuals through mass-administration of the only available drug praziquantel. However, the number of infected people has changed little over the last decades [1]. What is more, reduced susceptibility of *Schistosoma mansoni* worms to praziquantel has been reported in the field [2,3] and resistance to the drug can be induced under experimental conditions [4,5], raising the possibility that a similar situation could be also seen in the field. Consequently, the development of new mechanisms of intervention is a priority.

In this context, it is important that the process of infection is well characterised. The infectious agents for the human host, the cercariae, are microscopic free-living larvae released by infected fresh water snail hosts. Cercariae infect their mammalian host during water contact by trespassing across the skin barrier. This process is characterised by rapid morphologic, metabolic and physiological changes [6–9] that results in obligate parasitic schistosomula in as little as 2 hours [10]. The most prominent aspects of this transformation are the loss of the cercarial tail and a series of changes in the parasite’s surface. During skin penetration, the outermost layer in the parasite’s surface, the glycocalyx, gets thinner by the action of secretions from the parasite’s own acetabular glands [11], which are emptied during the process of transformation [12]. The remains of the glycocalyx are shed together with transient microvilli structures that form and disappear during this transformation process [13]. At the same time, pre-packed multi-laminated vesicles originating from the body of the parasite make their way to the surface where they release their contents; these contribute to the generation of the new double-bilayer membrane, characteristic of the intra-mammalian stage of the parasite [14].

Increasing research on the schistosomulum stage required the development of efficient, reproducible and rapid ways to generate large quantities of biological material. Various effectors are known to elicit the artificial transformation of cercariae into schistosomula, for example, cell growth media at 37 °C [15,16] or just low...
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Author Summary

Schistosomiasis is an endemic parasitic disease affecting ~200 million people in the most socioeconomically deprived regions of the world. Human infection occurs during water contact where free-living larvae called cercariae penetrate host skin and become parasitic organisms called schistosomula. This stage represents the first encounter of the parasites with the host and is also regarded as one of the most vulnerable stages of the parasite’s life cycle. Therefore, schistosomula are the focus of many studies, many of which look at changes in the expression of genes as a way of understanding the process of infection, identifying potential drug targets and vaccine candidates. Because collecting enough parasitic material from natural infections is not possible for certain types of studies (for example, gene expression studies), a mechanical transformation of the cercariae into schistosomula is often used instead and assumed as a good proxy for the natural transformation process. However, the equivalency of gene expression profiles between naturally transformed parasites and the mechanically transformed counterparts has never been studied. In this report, we analyse differences in gene expression patterns between these two different parasite preparations and provide enough data to resolve a long-lasting controversy.

osmolarity phosphate buffer saline solution [17] seem to be enough to trigger the cercariae to schistosomula transformation. The presence of certain skin lipids, yet is not essential [17], also plays a role in the process of cercariae transformation and penetration [18,19] probably by triggering the release of acetabular glands contents [20]. The most popular method for obtaining artificially transformed schistosomula uses a mechanical transformation (MT) protocol that includes some sort of shear force (centrifugation [21–23], passages through an emulsifying needle [24], or shaking [15]) applied to freshly shed cercariae followed by separation of cercariae heads from tails (usually by centrifugation in a density gradient) and posterior incubation of the cercariae heads/schistosomula in culture media at 37°C. Parasites obtained using this protocol show no major morphological or biochemical differences with those recovered from natural infections [10,15]; making the MT the method of choice for obtaining large quantities of schistosomula.

However, at the level of the whole transcriptome, equivalency of MT schistosomula to those obtained from natural infections has not been established; even though these artificial parasite preparations have been used almost exclusively in the identification of potential vaccine proteins and in high-throughput studies of gene expression [25–29], identification of drug targets [27] and screening of a compound library [30]. Artificial induction of stress or mechanical damage may induce gene expression signals that are not responding to the natural process of infection. Moreover, failure to induce physiologically important transcription events, triggered by host-skin specific signals, could lead to exploitable vulnerabilities being missed. Our work presented here uses high throughput transcriptome sequencing technology, known as RNA-seq [31], in combination with the latest genome assembly available for *S. mansoni* [32] to compare the profile of genes expressed in MT and ST schistosomula.

Materials and Methods

Biological material

*S. mansoni* (NMRI strain of Puerto Rican origin) cercariae were shed from infected Biomphalaria glabrata snails by exposing them to the light for 1.5 hours. MT schistosomula were obtained using an optimised version of the protocol used by Brink et al., [10]. Optimisation steps of the protocol were implemented in the tail detachment step (shake cercariae vigorously for approximately 30 seconds in a vortex mixer before passing these through a 21G syringe needle approximately 13–15 times) and the separation of heads/schistosomula and tails (by placing the heads plus tails suspension on 10 ml of ice-cold 70% Percoll (Sigma, UK) and 90 mM NaCl solution in DMEM in 15 ml conical tubes) by centrifugation at no more than 1000 g for 10 minutes at 4°C.

Skin-transformed (ST) schistosomula were obtained using a modified version of the protocol published by Clegg et al., [33]. TO (Tuck Ordinary) mice (Harland, UK) were killed with an overdose of anaesthetics followed by cervical dislocation according to Home Office regulations. Hair was removed from the abdominal and dorsal skin areas using clippers and skin was later excised from the animal using dissecting scissors. Each animal provided an area of skin of approximately 7.5 cm²; which was divided into two halves. Gel-like dermal tissue was removed by rubbing the skin gently (for approximately 5 minutes) with sterilized gauze soaked in supplemented DMEM (Dulbecco’s Modified Eagle’s medium supplemented with 100 U/L penicillin, 0.1 mg/L streptomycin and 10 mM L-glutamine). The transformation apparatus is presented in Figure 1A. The lower compartment of the assembly was filled with supplemented DMEM containing 2% fetal calf serum (FCS) and one half of prepared skin was mounted covering the opening of the tube with the dermal side facing downwards. The upper compartment was placed above the lower compartment with a rubber O-ring in between. All pieces were kept in place by holding both tubes with a metal clip (Figure 1B). The skin surface was washed three times with aquarium water and assemblies were checked for leaks. All assemblies were placed in a water bath pre-warmed at 37°C; the bottom compartment of the assembly was constantly kept at this temperature (Figure 1C). Experiments were carried out in a room with controlled temperature of 28°C. Approximately 12,000–14,000 freshly shed cercariae kept in aquarium water were placed in each assembly and these were left in the water bath for 3 hours. Schistosomula preparations were individually checked for contamination with tails. Samples with more than 4% tails/cercariae contamination were discarded.

MT and ST schistosomula preparations were placed in individual tubes, washed 3 times in supplemented DMEM and incubated at 37°C and 5% CO₂ for a total of 24 hours in growth media (supplemented DMEM, 10% FCS, 1% Hepes buffer). Schistosomula preparations were observed under the microscope using a Leica DM 1 L inverted microscope (Leica, Milton Keynes, Bucks, UK) at 10× or 40×. Video recordings were taken using a Dino-Lite AM-423X camera and DinoXcope software (Version 1.7.3). The criterion used for evaluating parasites is the one used in Mansour et al., [34]. After the incubation period was completed, parasites were transferred to 15 ml conical tubes and centrifuged at 1,000 g for 5 minutes, supernatant was discarded and schistosomula were suspended in 1 ml of TRizol reagent (Invitrogen, UK) and stored at ~8°C until RNA extraction.

RNA extraction, library preparation and sequencing

Total RNA from parasite material was extracted using TRizol (Invitrogen, UK) according to manufacturer specifications. After extraction, RNA quality was assessed using an Agilent RNA 6000 Nano - Bioanalyzer and quantified using a NanoDrop ND-1000 UV-Vis spectrophotometer. RNA-seq libraries were prepared as previously described [31] and sequenced as 76-base paired reads.
using the Illumina Genome Analyzer IIx platform. Raw sequence data were submitted to ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-451.

RNA-seq reads alignment and gene expression analysis

RNA-seq reads were aligned to the latest _S. mansoni_ reference genome (version 5.0, [32]) using TopHat [35] (version 1.3.1) with default parameters except for minimum and maximum intron sizes which were set to 10 and 30,000 bp respectively. Other parameters that were specified included the type of library sequenced (set to standard cDNA Illumina library; -library-type fr-unstranded) and the mate pair distance (or insert size; -r option), which was calculated individually for each library. Only uniquely mapping reads were kept for further analyses. The number of reads aligned to each transcript was calculated using BEDTools [36] and used to calculate RPKM (reads per kilobase per million of reads mapped) values [31] for each transcript. A threshold RPKM value was calculated as described in Protasio et al. [32], and transcripts with expression \( \leq 2 \) RPKM were removed from the dataset resulting in the reduction of the total number of transcripts from 11,778 to 9,291 (2,487 transcripts had negligible expression in both samples). Differential expression of transcripts was performed using EdgeR [37]. P-values were adjusted for multiple testing [38] and the threshold for significance set at adjusted \( p \)-value \( \leq 0.05 \). A complete list with fold change values and associated adjusted \( p \)-values obtained from EdgeR are provided in Supplementary Table S1.

RT-qPCR validation of differentially expressed genes

Relative expression of a subset of genes found differentially expressed between the ST and MT schistosomula were assayed using real time quantitative PCR (RT-qPCR). Primers for these genes were designed using Primer3 software [39] and ordered from Sigma, UK (primer sequences are available upon request). First strand cDNA was synthesised from 1 ug of original total RNA samples (MT2 and ST2 – Table 1) using SuperscriptII (Invitrogen, UK) according to manufacturers instruction. All RT-qPCR reactions were performed in a MX3005P QPCR System (Agilent Technologies) and using KAPA SYBR FAST qPCR Kit (Kapa Biosystems). PCR efficiencies for each primer pair were calculated using 10-fold dilutions of MT2 cDNA. Relative gene expression for a given gene was quantified relative to the expression of a reference gene (rRNA18S). Cycle thresholds (\( C_t \)) for each reaction where obtained using the MsPRO QPCR Software (Agilent Technologies) and used in the Pfaffl equation [40] to calculate the fold change expression of a target gene between samples. Fold change values reported are the mean of four replicates. In order to compare RT-qPCR and RNA-seq derived fold change values, RNA-seq standard deviation (SD) was calculated using the method described by Busby et al., [41].

Metabolic activity of schistosomula

AlamarBlue incorporates a colour indicator of metabolic activity of the mitochondrial function [42] and has previously been used to assess the viability of schistosomula [34]. In order to assess metabolic activity of MT and ST parasites, 250 24-hours-old-schistosomula obtained from MT and ST methods were incubated in AlamarBlue (Invitrogen, UK) for either 3 or 24 hours prior to measurement. Eleven and 12 samples were assayed for MT and ST respectively. Absorbance was measured at 570 nm (with reference at 600 nm) using a microplate reader BioTek PowerWave HT (BioTek Instruments Inc., Winooski, VT, USA); data collection was performed using the software Gen5 (BioTek Instruments Inc., Winooski, VT, USA). Raw absorbance data is presented in Supplementary Table S2. Student’s \( t \)-test was used to evaluate the significance between mean absorbance.

### Table 1. Summary of sequenced samples and alignment to the _S. mansoni_ reference genome.

| Sample | Replicate | Lane_id | Sequenced reads | Total reads mapped |
|--------|-----------|---------|-----------------|--------------------|
| MT 1   | 1         | 4912.5  | 50,616,612      | 61.80%             |
| MT 2   | 2         | 4912.6  | 50,441,286      | 57.87%             |
| ST 1   | 1         | 4912.7  | 44,496,358      | 53.13%             |
| ST 2   | 2         | 4912.8  | 48,970,182      | 57.50%             |
| Mean   |           |         | 50,834,175      | 57.57%             |

MT: mechanically transformed; ST: skin-transformed.

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Ethics statement
All animal procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and as authorised on personal and project licences issued by the UK Home Office.

Results
Optimization of transformation protocols
Both MT and ST transformation protocols were subjected to optimization. Results comparing the standard and optimised MT protocols are shown in Figure 2A and 2B respectively. For the MT, optimised conditions resulted in increased numbers of schistosomula and a lower percentage of damaged or non-viable individuals. Reduction of the number of syringe passages resulted in increased number of viable parasites while a higher percentage of Percoll resulted in less contaminating tails (~1%). For the ST, we found that schistosomula preparations virtually free from tail contamination (~1% to 4%) could be obtained by placing no more than 14,000 cercariae in the upper compartment of the transformation apparatus. However, tail contamination was more frequent in the ST preparations than in MT and samples dedicated for RNA-seq libraries had to be carefully selected. Contrary to schistosomula resulting from MT, non-viable parasites were hardly ever observed in ST preparations. Under the light microscope, schistosomula obtained from both protocols were indistinguishable from each other (Supplementary Video S1 and Video S2) and both schistosomula preparations progressed to later stages in the life cycle (up to two weeks post transformation) when cultured in vitro (data not shown). In terms of recovery, MT yields ~90% of the applied cercariae, while the ST yields only ~10%.

Differential gene expression between MT and ST schistosomula
An overview of the sequencing and alignment results obtained for the sequenced samples is presented in Table 1; where samples 1 and 2 (for both MT and ST) represent independent schistosomula transformations (replicates). In the case of ST samples, and due to the limited number of schistosomula obtained in each experiment, approximately 3 experiments were pooled to provide enough biological material.

Our dataset included 11,778 annotated transcripts and we found that 9,291 showed expression above background in at least one of the samples. Correlation analysis of the 24-hour old MT and ST schistosomula transcriptome samples showed high values for both Pearson’s product and Spearman’s rank coefficients (0.98 and 0.99, respectively; Figure 3A). Using the software package EdgeR [37], we found only 38 differentially expressed transcripts (adjusted p-value<0.05) between MT and ST schistosomula. Of these, 28 transcripts showed higher relative expression in the MT parasites (Table 2) while 10 transcripts showed higher relative expression in the ST (Table 3). A graphical representation of differentially expressed transcripts is shown in Figure 3B.

RT-qPCR validation was performed for 33 of the 38 genes found differentially expressed between MT and ST schistosomula (Figure 4). With 95% confidence interval, the fold change values obtained from both methods overlapped in 14 cases (Smp_029780, Smp_057860, Smp_124000, Smp_132670, Smp_172770, Smp_211020, Smp_212760, Smp_900010, Smp_900020, Smp_900030, Smp_900050, Smp_900070, Smp_900080, Smp_900090). Moreover, fold change values obtained from these two different methods are highly correlated (Pearson’s correlation 0.89, p-value 3.85E-12) and only in 4 cases (Smp_028850, Smp_067800, Smp_155320, Smp_001070) the direction of the fold change disagrees between the two methods.

Figure 2. Improving the mechanical transformation protocol. A – Parasites were transformed according to a standard protocol [10] where parasites are subjected to 23 passages through a syringe needle and separation of heads and tails is performed with 60% Percoll solution at room temperature. B – Transformation of parasites was performed using the optimized protocol described in Material and Methods; which involved shaking of the cercariae suspension in a vortex mixer followed by only 12–13 passages through a syringe needle. Separation of schistosomula/heads from tails was performed using 70% ice-cold Percoll solution. Arrows indicate damaged schistosomula, arrowheads point to contaminating cercariae or tails. Both panels (A and B) show light microscope images of 3-hours post-transformation schistosomula.

Genes more highly expressed in ST schistosomula
Table 2 shows a list of genes more highly expressed in skin-transformed parasites. We found that all 12 mitochondrial genes (Smp_900000–Smp_900110) are found in this list. In order to investigate whether the higher expression of the mitochondrial genes had any consequences on metabolic activity we used the AlamarBlue (AB) assay. AB is a good indicator of mitochondrial activity through the measurement of redox species generated by the respiratory electron chain [42]. Incubation of 24-hour old schistosomula for 3 hours in AB showed no significant difference between MT and ST parasites (Figure 5 – blue boxplots). Increasing the incubation time to 24 hours showed an incremental increase in the absorbance (compared to blank wells) and a significant difference (t-test, p-value<0.01) between MT and ST schistosomula (Figure 5 – green boxplots) suggesting that these two populations of parasites have not only different rates of mitochondrial metabolism but that ST parasites are more
metabolically active than their MT counterparts after 24 hours of in vitro culture.

The remainder of genes found relatively more expressed in the ST sample included examples that could be associated with the infection process. Two of these genes, for instance, are involved in calcium sensing (Smp_151600) or binding (Smp_132670), functions that have been associated with schistosomula adaptation to the mammalian host [43]. It is possible that such mechanisms are induced by contact with the host skin explaining the reduced expression of such transcripts in MT parasites.

**Genes more highly expressed in MT schistosomula**

Proteases and protease inhibitors are among the transcripts that were more expressed in MT schistosomula. Proteases have a recognised role in schistosomes; for example, adult worms use a set of aspartic proteases called cathepsins for the purpose of feeding [44] while cercariae use elastase and other proteases during the process of skin invasion [45,46]. We found a gene encoding a secreted serine protease from the trypsin family (Smp_002150), with expression in MT parasites double that of ST parasites. RNA-seq data (Supplementary Table S3 in Ref. [32]) showed that the expression of this gene is developmentally up regulated after the transformation in schistosomula, showing an impressive 30-fold increase between 3-hour and 24-hour post-transformation parasites.

Alongside the serine protease we found two protease inhibitors that were also differentially expressed. Protease inhibitors can neutralise the action of host- and/or parasite-derived proteases. Smp_089670 encodes a 1,800 amino acids polypeptide with high similarity to an alpha-macroglobulin. Macroglobulin-type inhibitors entrap their target proteases limiting the range of substrates they can act upon; hence, they have a regulatory role rather than strictly inhibitory effect [47]. Macroglobulins can also inhibit coagulation [48], perhaps indicating that the secretion of the *S. mansoni* alpha-macroglobulin functions as a facilitator of schistosomula migration through the broken tissue/vessels during the skin stage. The second protease inhibitor was a kunitz-type serine protease inhibitor (Smp_147730). These types of inhibitors have been postulated to have an important role in the host-parasite interaction in *Echinococcus granulosus* infections [49]. Interestingly, both protease inhibitors were significantly up regulated during transformation from cercariae to schistosomula (Supplementary Table S3 in Ref. [32]) suggesting that their expression is developmentally regulated.

**Two microexon genes are more expressed in MT schistosomula**

Microexons (<36 bp, in multiples of 3 bases) typically form a small part of some genes in most eukaryotes [50] but for a few genes in *S. mansoni*, microexons comprise the majority (~75%) of the sequence length and these genes have therefore been termed microexon genes (MEGs) [51,52]. Each MEG has the potential to generate an enormous repertoire of splicing variants through exon skipping because missed exons do not cause frame-shifts. The particular gene structure of MEGs therefore provides an easy mechanism to generate protein variation and seems to be both time and tissue specific [52].

Two MEGs from two different families appeared more expressed in MT compared to ST schistosomula at 24 hours after transformation (Table 3). In the case of Smp_180340, a MEG-2 member, RNA-seq coverage was poor and not specific to the exons in both samples; probably indicating unprocessed transcripts and was not considered for further analysis. For Smp_124000, RNA-seq data from schistosomula samples agreed with the current annotation of the gene (Figure 6). Intriguingly however, the isoforms expressed in MT and ST differed from each other, with three exons that were expressed in the ST being absent from the MT schistosomula sample (Figure 6). Because the RNA-seq experiments assayed the transcriptional status of large numbers of parasites simultaneously, we can therefore rule out – at least in this example – that exon skipping is simply a stochastic process.

Figure 3. Skin-transformed (ST) and mechanically-transformed (MT) schistosomula are transcriptionally very similar. A – Correlation of gene expression values between MT (x-axis) and ST (y-axis) schistosomula. Both Pearson and Spearman’s correlations are high (0.98 and 0.99 respectively) indicating very low variability between these two samples. B – Differential gene expression (MA plot) between MT and ST parasites at 24 hours after transformation (adjusted p-value<0.05). Relative concentration (x-axis) is plotted against fold change values (y-axis) in the log₂ scale. Positive log₂ fold changes represent transcripts more expressed in ST schistosomula while negative log₂ fold changes represent transcripts more expressed in MT schistosomula.

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### Table 2. Genes with higher expression in skin-transformed schistosomula.

| Gene identifier | Fold change | Adj. P-value | Gene product description                                      |
|-----------------|-------------|--------------|----------------------------------------------------------------|
| Smp_900100      | 9.25        | 1.57E-32     | NADH dehydrogenase subunit 3                                  |
| Smp_204970      | 5.06        | 1.23E-03     | Hypothetical protein                                           |
| Smp_172770      | 4.76        | 6.73E-03     | Hypothetical protein                                           |
| Smp_900040      | 3.43        | 5.68E-18     | NADH dehydrogenase subunit 2                                  |
| Smp_900020      | 3.41        | 7.30E-18     | NADH dehydrogenase subunit 6                                  |
| Smp_900110      | 3.36        | 1.94E-17     | NADH dehydrogenase subunit 1                                  |
| Smp_029780      | 3.34        | 4.77E-02     | Hypothetical protein                                           |
| Smp_900060      | 3.16        | 9.52E-16     | Cytochrome c oxidase subunit III                              |
| Smp_900050      | 3.14        | 1.39E-11     | NADH dehydrogenase subunit 5                                  |
| Smp_028850      | 3.03        | 1.30E-13     | Hypothetical protein                                           |
| Smp_127860      | 2.85        | 3.82E-04     | Fmrfamide receptor                                             |
| Smp_202120      | 2.85        | 1.05E-06     | Hox class homeodomain protein djabd bb;                      |
| Smp_900090      | 2.79        | 1.33E-12     | NADH dehydrogenase subunit 4                                  |
| Smp_067800      | 2.64        | 9.45E-16     | Fibrillin 2                                                    |
| Smp_900300      | 2.51        | 5.14E-10     | ATP synthase F0 subunit 6                                     |
| Smp_900007      | 2.5         | 7.99E-10     | Cytochrome B                                                  |
| Smp_900010      | 2.45        | 1.94E-09     | Cytochrome c oxidase subunit II                               |
| Smp_900080      | 2.39        | 3.71E-13     | NADH dehydrogenase subunit 4L                                 |
| Smp_170630      | 2.33        | 4.57E-02     | Periostin, putative                                            |
| Smp_146760      | 2.31        | 6.40E-04     | Hypothetical protein                                           |
| Smp_900000      | 2.3         | 3.92E-08     | Cytochrome c oxidase subunit I                                |
| Smp_153220      | 1.97        | 1.43E-04     | Hypothetical protein                                           |
| Smp_212760      | 1.89        | 1.97E-06     | Kinesin, putative                                             |
| Smp_151600      | 1.87        | 2.00E-02     | Neuronal calcium sensor 2                                     |
| Smp_057860      | 1.78        | 3.08E-03     | Hypothetical protein                                           |
| Smp_132670      | 1.77        | 2.75E-03     | Myosin regulatory light chain 2 smooth muscle                 |
| Smp_001070      | 1.59        | 5.51E-03     | Hypothetical protein                                           |
| Smp_211020      | 1.59        | 6.39E-03     | Cell adhesion protein                                          |

Differentially expressed genes with adjusted p-value < 0.05 are shown. Gene identification numbers with a prefix “Smp_90” are those encoded in the mitochondrial genome.

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### Table 3. Genes with higher expression in mechanically transformed schistosomula.

| Gene identifier | Fold change | Adj. P-value | Gene product description                                      |
|-----------------|-------------|--------------|----------------------------------------------------------------|
| Smp_180340      | 6.06        | 2.33E-02     | MEG-2 (ESP15) family                                          |
| Smp_113660      | 4.63        | 4.57E-03     | U1 small nuclear ribonucleoprotein C                           |
| Smp_147730      | 2.57        | 1.19E-05     | Single kunitz protease inhibitor; serine type protease inhibitor|
| Smp_203400      | 2.45        | 1.77E-02     | Rhodopsin orphan GPCR                                          |
| Smp_204260      | 2.08        | 1.11E-02     | Mastin                                                         |
| Smp_002150      | 1.93        | 2.86E-07     | Mastin, subfamily 51A unassigned peptidase (501 family)        |
| Smp_089670      | 1.88        | 1.32E-06     | Alpha 2 macroglobulin                                          |
| Smp_063330      | 1.73        | 6.73E-03     | Hypothetical protein                                           |
| Smp_124000      | 1.60        | 2.84E-02     | MEG-14                                                         |
| Smp_070240      | 1.54        | 1.08E-02     | Venom allergen-like (VAL) 7 protein                            |

Differentially expressed genes with adjusted p-value < 0.05 are shown.

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Discussion

The process of cercarial invasion and early stages of schistosomula migration are relevant for the development of intervention strategies against schistosomiasis. The skin schistosomula stage represents the first encounter of the parasite with the mammalian host and it is regarded as a vulnerable stage for parasite killing [53–55]. Hence, schistosomula have been the target of many studies that focus on both the adaptation of the parasite to its host and the identification of drug targets and vaccine development.

The study of changes in gene expression across different stages of host invasion can be used to investigate parasite adaptation to the host. With one exception, where in vivo recovered and in vitro cultured S. japonicum schistosomula were compared [56], all high-throughput gene expression studies have used MT schistosomula at different developmental stages by just prolonging the in vitro incubation time [25,27,28,32,57]. Since MT schistosomula are only proxies for natural infections, the differences between these and more naturally transformed parasites needs to be established. For instance, misleading artefactual parasite responses induced by stress or damage need to be identified as well as potentially important parasite responses that are only induced during the rapid natural transformation of free-living cercariae into obligatory parasitic schistosomula. Our work used the latest RNA-seq technology to investigate the differences in the gene expression of MT and ST schistosomula at 24-hour post-transformation. We found that these samples differ only in the expression of 38 transcripts (out of 9,291 expressed transcripts; adjusted \( p \)-value<0.05). In order to validate our approach, we performed RT-qPCR on 33 out of the 38 genes found differentially expressed and found that, at least in this experiment, RNA-seq seems to overestimate the fold change values of differentially expressed genes. However, a high correlation value of 0.89 was found between the two methods (similar values have been reported elsewhere [58]) suggesting that the RNA-seq is a valid and reliable method for high-throughput identification of differentially expressed genes. Increased transcript coverage (greater sequencing depth) as well as the addition of more biological replicates may result in better measurement by providing greater statistical power.

Transcripts encoded in the mitochondrial genome (mitochondrial genes) were found more highly expressed in ST parasites resulting in higher metabolic rates in ST parasites. Previously Brink et al., [10] suggested that ST parasites are a selection of the most “fit” cercariae. We suggest that MT preparations may contain a mixture of fit and less-fit parasites and therefore not all of them are expected to develop at their maximum metabolic rate resulting in an averaged reduced metabolic activity for the MT schistosomula population.

MT parasites showed higher expression of a protease and two protease inhibitors. Proteases have been linked to host tissue invasion.
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The exons or lack of exons found differentially expressed in our analysis could be associated with the functions described in the S. japonicum study; probably due to the differences in the experimental design of both studies (i.e., time points at which differential expression is assessed).

Our study represents a snapshot of the schistosomula transcriptome after transformation. It is possible that a greater effect of the different treatments applied to both populations might be seen at shorter times after transformation. Ideally, a time-course experiment comprising more than one time point comparison between ST and MT schistosomula should have been performed. Nevertheless, should differences in gene expression exist at earlier time points, they disappear at 24 hours after transformation and are unlikely to have consequences on the gene expression profile of the parasites.

Finally, we emphasise that in view of the great differences in the transformation processes analysed here, the number of genes found differentially expressed between ST and MT at 24-hours role and regulation of alternate splicing in MEG may become clearer as the functions of microexon genes are further elucidated.

Due to the different treatments received by MT and ST schistosomula, including the low temperature and mechanical stress endured by MT schistosomula, we had hypothesised that stress-associated transcripts (e.g. stress/apoptotic pathways) would be differentially expressed. Surprisingly, we could not identify clear markers of stress, possibly because we had optimised our MT protocol to yield a minimum proportion of damaged parasites. Other MT protocols involving, for example a greater number of passages through a syringe-needle or a different source of mechanical stress may give different results.

Since MT schistosomula are not exposed to skin lipids that are known to play a role in transformation [18,19] and induce the release of contents from the acetabular glands [20], we had anticipated observing differences related to the presence of lipids in the ST. However, we could not identify transcripts related to the binding (fatty-acid binding proteins) or to the transport of fatty acids and therefore conclude that the effect elicited by the presence of skin lipids is independent from transcriptional regulation at that time and is more likely related to machinery that the parasite may already have in place prior to its encounter with host skin.

Previously, gene expression changes using in vivo recovered (IVS) and mechanically transformed schistosomula (MTS) in S. japonicum at 3 days after transformation has been published [56]. The authors showed that IVS parasites show higher expression of transcripts encoding protaglandins, glutathione-S-transferase Sm29GST, paramyosin, stress related proteins and transcripts related to markers of anti-inflammatory and immunomodulatory processes. In the case of MTS parasites, the authors report higher expression of transcripts involved in glucose transport, fatty acids transport and haemoglobin digestion. None of the genes found differentially expressed in our analysis could be associated with the functions described in the S. japonicum study; probably due to the differences in the experimental design of both studies (i.e., time points at which differential expression is assessed).

Our study represents a snapshot of the schistosomula transcriptome after transformation. It is possible that a greater effect of the different treatments applied to both populations might be seen at shorter times after transformation. Ideally, a time-course experiment comprising more than one time point comparison between ST and MT schistosomula should have been performed. Nevertheless, should differences in gene expression exist at earlier time points, they disappear at 24 hours after transformation and are unlikely to have consequences on the gene expression profile of the parasites.

Finally, we emphasise that in view of the great differences in the transformation processes analysed here, the number of genes found differentially expressed between ST and MT at 24-hours

Figure 5. Mitochondrial activity is higher in ST schistosomula. AlamarBlue reactivity of 24-hour old MT and ST schistosomula incubated for either 3 hours (blue) or 24 hours (green). Longer incubation time in AlamarBlue provided the necessary resolution to establish a significant difference (t-test, p-value <0.01, indicated with *) in metabolic activity between the mechanically (M) and skin (S) transformed schistosomula; (B) Blank wells.

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Figure 6. The microexon gene Smp_124000 is expressed as different isoforms in the ST and MT schistosomula. The exons or lack of them that contribute to the new isoform are marked in dashed boxes while exons that are differentially expressed between the ST and MT schistosomula are marked with a star (*).

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post-transformation is unexpectedly small, suggesting that changes in gene expression induced upon transformation might be independent from the methodology employed to transformed parasites, at least in the two methods here studied.

In this work, we provide further evidence that transformation might be triggered by more robust signals, such as the change in osmotic pressure [17] and/or temperature [15] between the water and the host environments. We recommend that, except for the reported genes, these samples should be considered as transcriptionally equivalent. Our work contributes to the validation of gene expression studies that have used MT schistosomula and provides further evidence that the MT is a good proxy for natural skin-transformation.

Supporting Information

Table S1 | Complete list of differentially expressed genes. Fold change values, p-values and adjusted p-values are shown for each transcript as reported by EdgeR [37].

Table S2 | Raw absorbance values collected for MT and ST samples during 3- and 24-hours incubation in Alamar Blue.

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Video S1 | This supporting information file shows schistosomula obtained using the optimised mechanical transformation protocol described in Materials and Methods. (MP4)

Video S2 | This supporting information file shows schistosomula obtained using the skin transformation [33]. (MP4)

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Author Contributions

Conceived and designed the experiments: AVP DWD MB. Performed the experiments: AVP. Analyzed the data: AVP. Contributed reagents/materials/analysis tools: DWD MB. Wrote the paper: AVP DWD MB.
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