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Effect of human TGF-β on the gene expression profile of *Schistosoma mansoni* adult worms

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ABSTRACT

Schistosoma mansoni is responsible for schistosomiasis, a parasitic disease that affects 200 million people worldwide. Molecular mechanisms of host–parasite interaction are complex and involve a crosstalk between host signals and parasite receptors. TGF-β signaling pathway has been shown to play an important role in *S. mansoni* development and embryogenesis. In particular human (h) TGF-β has been shown to bind to a *S. mansoni* receptor, transduce a signal that regulates the expression of a schistosome target gene. Here we describe 381 parasite genes whose expression levels are affected by in vitro treatment with hTGF-β. Among these differentially expressed genes we highlight genes related to morphology, development and cell cycle that could be players of cytokine effects on the parasite. We confirm by qPCR the expression changes detected with microarrays for 5 out of 7 selected genes. We also highlight a set of non-coding RNAs transcribed from the same loci of protein-coding genes that are differentially expressed upon hTGF-β treatment. These datasets offer potential targets to be explored in order to understand the molecular mechanisms behind the possible role of hTGF-β effects on parasite biology.

1. Introduction

Schistosomes are complex trematode parasites that have six developmental stages and two hosts: a snail as intermediate host belonging to genus *Biomphalaria* and a vertebrate as definitive host. They cause schistosomiasis, a serious parasitic disease worldwide, with an estimated 200 million people afflicted in 76 countries and territories located in tropical and subtropical areas [1].

The parasites have a highly adapted relationship with their hosts that appears to involve the schistosome exploitation of host endrocrine and immune signals, in addition to nutrient uptake, for their development and differentiation [2–6]. Recent description in the parasite of homolog genes to vertebrate receptors suggests that schistosomes are potentially responsive to host molecules [7–13], and as a consequence the role of host factors in schistosome development has been explored [4,14–17].

Transforming growth factor beta (TGF-β) is a cytokine that regulates many processes central to life of metazoans such as growth and differentiation, developmental patterning, tissue repair and cell death [18]. The basic signaling pathway of TGF-β comprises the TGF-β receptors type I and type II that are structurally similar transmembrane serine/threonine kinases; SMADs proteins: R-Smads (e.g., Smad 1/Smad 2 in vertebrates) that interact with the receptors, are phosphorylated by receptors and then will associate with other SMADs, co-SMADs (Smad4) to form a transcription regulatory complex that translocates to the nucleus. Through interaction of co-regulators such as CBP (CREB binding protein) or p300, the complex activates/represses transcription of target genes [18,19].

TGF-β signaling elements have been described in *Schistosoma mansoni*; two types of TGF-β receptors I (SmTβR1) and II (SmTβR1) [9]; four SMADs: SmSMAD1 [20], SmSMAD1b [21], SmSMAD2 [22], SmSMAD4 [23]; one homolog gene to Inhinbin/Activin SmInAct [24]; a homolog to BMP (Bone Morphogenic Protein), SmBMP [25] and Smp300/CBP was also identified [26]. It has been shown that these elements can act together in the signaling process in *S. mansoni* and play an important role in the development of vitelline cells in female worms and in embryogenesis of eggs by male stimuli [24,27–29].

In spite of all the evidence of a TGF-β effect in parasite biology, the molecular basis to understand which genes are affected at the transcriptional level have not until this study been explored. In

Abbreviations: AMPK, 5′AMP-activated protein kinase; BMP, bone morphogenic protein; CBP, CREB binding protein; eIF, eukaryotic translation initiation factor; Erk, extracellular signal-regulated kinases; GEO, gene expression omnibus; GO, Gene Ontology; hTGF-β, human transforming growth factor-β; miRNA, microRNA; ncRNAs, non-coding RNAs; R-SMAD, receptor-mediated SMAD; RT-qPCR, reverse transcription-quantitative real time PCR; Sm, *Schistosoma mansoni*; SYK, spleen tyrosine kinase.

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this work we present the effect of human TGF-β on the gene expression profile of adult worms, resulting in increased knowledge about host–parasite molecular cross talk.

2. Materials and methods

2.1. Parasite treatment and RNA extraction

Schistosoma mansoni (NMRI) maintained in a Puerto Rican *Biomphalaria glabrata* and Golden Syrian hamsters was used for these studies. Parasites were recovered by portal perfusion of infected hamsters 45 days after infection [30].

Freshly perfused adult worms were washed in M169 medium plus antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin, 1 mg/ml amphotericin B (antibiotic antimycotic solution) and cultured 22–24 h in M169 medium supplemented with 10% Fetal Bovine Serum and antibiotics). 1 nM of recombinant human TGF-β (R&D Systems, Inc.) was added and the worms cultured for 22–24 h. We performed three biological replicas of treated worms (annotated as TGF_1, TGF_4 and TGF_5) and two biological replicas of control non-treated parasites (Ctrl_2 and Ctrl_3).

RNA from each sample was extracted using Trizol reagent (Invitrogen), and then treated with DNAse using RNAeasy kit (QIAGEN) according to the manufacturerĩs instructions. RNA integrity was evaluated using Bioanalyzer microfluidic electrophoresis (Agilent Technologies).

2.2. Microarray experiments

We used 300 ng of each sample to perform linear amplification using low input RNA amplification kit (Agilent Technologies); for each TGF treated sample (TGF_1, TGF_4 or TGF_5) we performed 4 technical replicas. The control samples (Ctrl_2 and Ctrl_3) were pooled together (using the same amount of RNA) and were used for each amplification reaction. In a similar manner, we performed 12 technical replicas of the control pool (to combine to each one of the four technical replicas from three biological samples). Treated samples were labeled with Cy3 or Cy5 dye, control samples were labeled with the opposite dye and 825 ng of each labeled sample was combined for hybridization on a microarray slide, in a dye-swap approach.

Hybridization was carried out overnight on a custom 4×44 k oligonucleotide array designed by our research group [31] and produced by Agilent Technology following the manufacturer's instructions. Detailed description of 4×44 k platform is found in [31] and the platform probe annotation is available on gene expression omnibus (GEO) under the accession number GPL8606.

Slides were washed and scanned according to Agilent instructions using GenePix 4000B scanner (Molecular Devices). Raw data were extracted using Feature Extraction software (Agilent Technologies). Raw data is available in GEO under the accession number GSE27050.

We kept in the analysis genes that have a significantly detectable signal (using the IsPosAndSig column from Feature Extraction data output) in at least 75% of all replicas in at least one biological condition (treated or control). The intensities were normalized by LOWESS algorithm [32] and the log 2 ratio between treated and control was calculated. Under our experimental conditions, biological and technical variability were in the same range as evaluated by the intensity signal Pearson correlation coefficients: for the three biological replicas of Treated samples the average correlation = 0.901 (range = 0.837–0.939), whereas for the technical replicas of these Treated samples the average correlation = 0.951 (range 0.904–0.975). For the technical replicas of Control samples the average correlation = 0.983 (range 0.971–0.996).

To find the differentially expressed genes between treated and control we used SAM (Significance Analysis of Microarray) approach [33], and genes with a q-value <0.05 and were selected as differentially expressed. Next, we used the leave-one-out [34] statistical approach to find genes that are consistently differentially expressed between treated and control; we selected as differentially expressed those genes that have a q-value lower than 0.05 in all possible comparisons between two biological replicas (TGF_1 and TGF_4; TGF_1 and TGF_5; TGF_4 and TGF_5). Finally, we applied a cutoff filter to keep as differentially expressed only those genes with an average $|\log 2 \text{ ratio (treated/control)}| \geq 1.0$ (i.e., minimum two fold increase or decrease in gene expression in TGF-β treated in relation to control worms).

Analysis of Gene Ontology (GO) was performed using Ontologizer [35], and the p-value was adjusted using Benjamin–Hochberg method [36]. Functional analysis was performed using Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com/). For this purpose we annotated *S. mansoni* genes encoding putative homologs to human proteins; the putative homolog should have similarity with a BlastX e-value lower than 1×10^{-10} and coverage of at least 60% of the human homolog. The RefSeq number of each human homolog was associated to each *S. mansoni* gene and the expression data was uploaded to Ingenuity Pathway Analysis System version 7.6. We included all gene/protein relationships described as experimentally observed and/or predicted with high confidence.

2.3. RT-real time PCR experiments

In order to confirm the expression pattern of some candidates, we performed RT-real time PCR experiments. Five hundred ng of RNA from each sample was used for reverse transcription using QuantiTect reverse transcription kit (QIAGEN) following the manufacturerĩs instructions. In parallel, 500 ng of RNA from each sample was also incubated in reaction medium without reverse transcriptase enzyme as a negative control.

From the previous step, 0.15 μl of the reverse transcription reaction was used to perform Real Time PCR using Power SYBR Green Master Mix (Applied Biosystems) in the 7500 Real Time PCR System (Applied Biosystems) using the delta-CT method default parameters. Primer express software (Applied Biosystems) was used to design primers for selected genes, and the list of primers used is available in Supplementary Table 1. Data was analyzed according to the comparative CT method [37], Student t-test was used to calculate the significance. *S. mansoni* alpha-tubulin (sat-1, gi|161071, M80214.1) was used as the reference gene; we performed an absolute qPCR determination (with primers described in Supplementary Table 1) to evaluate sat-1 expression and the result is shown in Supplementary Fig. 1. There was no detectable Ct changes between hTGF-β treated worms and control worms.

3. Results

3.1. Identification of differentially expressed genes and functional analysis of protein coding genes

Human TGF-β (hTGF-β) at a concentration of 1 nM has been shown to affect *S. mansoni* adult worms, when parasites are incubated overnight in culture [9]. We used similar treatment conditions and total RNA was extracted from parasites. mRNA was linearly amplified and labeled as described under Section 2, and used for microarray hybridization experiments with an oligoarray platform containing 44-thousand gene probes [31].

We identified 8649 expressed genes, for which the intensity signals were significantly above the background in at least 75% of the