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Characterization of microRNA Expression Profiles in *Leishmania* Infected Human Phagocytes

Nicholas S. Geraci¹, John C. Tan^{1,2}, and Mary Ann McDowell¹

¹Department of Biological Sciences, Eck Institute for Global Health, University of Notre Dame, Notre Dame, Indiana, United States

²Genomics and Bioinformatics Core Facility, University of Notre Dame, Notre Dame, Indiana, United States

Abstract

Leishmania are intracellular protozoa that influence host immune responses eliciting parasite species specific pathologies. MicroRNAs (miRNA) are short single stranded ribonucleic acids that complement gene transcripts to block protein translation and have been shown to regulate immune system molecular mechanisms. Human monocyte derived dendritic cells (DC) and macrophages (MP) were infected *in vitro* with *Leishmania major* or *Leishmania donovani* parasites. Small RNAs were isolated from total RNA and sequenced to identify mature miRNAs associated with leishmanial infections. Normalized sequence read count profiles revealed a global down-regulation in miRNA expression among host cells following infection. Most identified miRNAs were expressed at higher levels in *L. donovani* infected cells relative to *L. major* infected cells. Pathway enrichments using *in silico* predicted gene targets of differentially expressed miRNAs showed evidence of potentially universal MAP kinase signaling pathway effects. Whereas JAK-STAT and TGF- β signaling pathways were more highly enriched using targets of miRNAs up-regulated in *L. donovani* infected cells. These data provide evidence in support of a selective influence on host cell miRNA expression and regulation in response to differential *Leishmania* infections.

Keywords

Leishmania; Leishmaniasis; microRNA; miRNA; dendritic cell; macrophage

INTRODUCTION

Leishmaniasis comprises a group of neglected diseases caused by obligate, intracellular protozoan parasites of the Genus *Leishmania*. These parasites are transmitted by the bites of

CORRESPONDENCE Mary Ann McDowell, Department of Biological Sciences, University of Notre Dame, Galvin Life Science Center, Notre Dame, Indiana, USA 46556 (mcdowell.11@nd.edu).

DISCLOSURES

None

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

sand fly vectors and distributed within tropical and sub-tropical regions. *Leishmania* reside within vertebrate host phagocytes, primarily dendritic cells (DC) and macrophages (MP) (1). Clinical manifestations of leishmaniasis are classified into three main forms: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL). Each species is uniquely associated with a distinct disease pathology. *Leishmania major* is one primary Old World parasite responsible for CL, which is characterized by ulcerative lesions localized at the point of inoculation (2, 3). Also referred to as kala-azar, VL is the most severe form of the disease, resulting from infection by species of the *L. donovani* spp. complex. These species disseminate throughout host vasculature and infiltrate visceral organs including the spleen, liver, and lymph nodes, leading to a variety of potentially fatal responses including spleno- and hepatomegaly, pancytopenia, weight loss, and high fever (3, 4).

Comparative microarray studies on the effects of *L. major* and *L. donovani* upon gene expression in bone marrow derived MPs from BALB/c mice (5) and monocyte-derived DCs (MDDC) and MPs (MDMP) from humans (6) revealed similarities between host gene expression patterns in response to either parasite species within the same host cell type. The latter study showed a greater diversity of differentially affected gene expression profiles in MDDCs, especially among IL-12 associated genes, NF κ B and IFN- γ pathway associated transcripts. These results indicate *Leishmania* species-specific molecular mechanisms influencing DC responses. However, the regulatory mechanisms governing the translation of those gene expression profiles are not fully elucidated.

Key for connecting disparate gene expression and molecular immunological pathway data is to understand changes in the multi-faceted regulation of those pathways. MicroRNAs (miRNA) are untranslated single-stranded RNA molecules of 18-26 nucleotides (nt) in length. MicroRNAs are part of a major system of molecular regulation referred to as RNA interference (RNAi) and have been identified in a wide variety of organisms including humans (7, 8). Mature miRNAs complement 'target' messenger RNA transcripts (mRNA), permitting transcript degradation and blockade of protein translation (9-11). It is estimated that about one-third of all human genes are under the post-transcriptional regulatory control of miRNAs, with each miRNA having about 100-200 potential transcript targets (12). While most miRNAs play roles in homeostasis, collectively, miRNAs target transcripts part of highly complex molecular pathways (13), such as are commonly found in immunobiology. We present here, a study of mature miRNA expression patterns in *L. major* and *L. donovani* infected human DCs and MPs using total small RNA and next generation sequencing (NGS). Our data demonstrate unique mature miRNA expression profiles in response to both parasite species in different human host cell types.

MATERIALS AND METHODS

Monocyte Derived Dendritic Cells and Macrophages

CD14⁺ monocytes of four anonymous healthy adult female human donors (Central Indiana Regional Blood Center, Indianapolis, IN) were isolated from peripheral blood mononuclear cells by positive selection using an AutoMACS separator (Miltenyi Biotec) after isolation using lymphocyte separating media (Cellgro) following manufacturer protocols. The purity

of separated monocytes was determined by flow cytometry analysis on a MCL500 flow cytometer (Beckman Coulter) using fluorescent antibody staining for CD14, CD19, CD45, and CD3 (Biolegend). Enriched monocytes destined for differentiation into DCs were treated with granulocyte-macrophage colony-stimulating factor (GM-CSF; 2,000 U/mL) and interleukin-4 (IL-4; 800 U/mL) recombinant cytokines (Peprotech) on days 0, 3, and 6 after plating in six-well plates at 2.5×10^6 cells/mL in complete RPMI medium (10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine). Enriched monocytes destined for differentiation into MPs were treated with macrophage colony-stimulating factor (M-CSF; 500 U/mL) recombinant cytokines (Peprotech) on days 0, 3, and 6 after plating in six-well plates at 2.5×10^6 cells/mL in complete RPMI medium. Immature DC were harvested on day 7 and plated in 6-well plates at 2×10^6 cells in 2 mL complete RPMI medium per well in the absence of exogenous cytokine. Similarly, medium containing exogenous cytokines was removed from MP cultures on day 7 and replaced with fresh complete RPMI medium without cell removal to avoid cellular activation by mechanical scraping. The phenotype purities of MDDCs and MDMPs were determined similarly by flow cytometry analysis of CD1a, CD14, HLA-DR, CD40, CD80, and CD86 expression.

Parasites and Infections

All infections were performed with *L. major* Friedlin V1 strain (MHOM/IL/80/Friedlin) and *L. donovani* 1S strain (MHOM/SD/00/1s). All parasite strains were cultured at 26°C without CO₂ in complete medium 199 (20% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 40 mM HEPES, 0.1 mM adenine in 50 mM HEPES, 5 mg/mL hemin in 50% triethanolamine, and 1 mg/mL biotin). Parasites tested negative for mycoplasma using a Mycoplasma PCR detection set (Takara) and tested below the detection limits for endotoxin using a Limulus Amoebocyte Lysate kit (Charles River Laboratories). Infective-stage metacyclic promastigotes were isolated by use of a Ficoll gradient as previously described (14) and opsonized with 5% normal human serum. Parasites were applied to host cells in culture at a multiplicity of infection (MOI) of 10:1. After eight hours post-infection, MDDCs were harvested washing in medium and application of 0.5 mM EDTA (Gibco) in 1X PBS (Cellgro). MDMPs were harvested at the same time by scraping and washing in medium. Cell harvests were spun down, washed, and pellets resuspended in RNeasy RNA Stabilization Solution (Applied Biosystems). Infection rates were determined at the end of each experiment by DiffQuick staining of cytospin whole cell preparations and visualization by light microscopy, where between 43% and 96% of cells were infected with infection indices of 3 to 15. No significant differences were observed between infection contexts or cell types across the four donors (data not shown).

RNA Isolation

For miRNA expression profiling, total RNA was isolated using the mirVana miRNA Isolation Kit (Ambion) following manufacturer instructions, purified using DNase I (Invitrogen) digestion, and recovered by acid phenol:chloroform. Total RNA was electrophoresed on a 15% TBE urea pre-cast gel (Novex), following manufacturer instructions, alongside flanking microRNA markers (New England Biolabs), and stained with SYBR Gold Nucleic Acid Gel Stain (Life Technologies). Gel sections of RNA samples

in the range of 17 to 25nt were cut using clean instruments and small RNA was extracted from the sections by diffusion as previously described (15). For gene transcript expression assays, large RNA was isolated with the RNeasy Mini Kit (Qiagen), purified using DNase I (Invitrogen) digestion, following manufacturer instructions with spin columns that do not retain RNA shorter than 70nt in length.

Next Generation Sequencing

Following quality analysis using Small RNA Analysis Kits on a 2100 Bioanalyzer (Agilent), small RNA samples, size separated by denaturing gel electrophoresis, were prepared for sequence analysis by successive 3' and 5' adapter ligations and generation of cDNA, as previously described (16), to extend the sequences to accommodate a 100 bp minimum length requirement. Samples were run on a 454 Genome Sequencer FLX+ (Roche) using 16-region nanowell sequencing plates with Titanium II chemistry per manufacturer instructions. Sequence read data were quality filtered and adapter trimmed by in-house programs.

Quantitative Real Time PCR (qRT-PCR)

Large RNA was reverse transcribed using random primers with the Superscript III synthesis system (Invitrogen) for RT-PCR according to the manufacturer instructions. Primers to specific gene products were designed with sequences obtained at GenBank (NCBI) and using Primer Quest (IDT) and Primer BLAST (NCBI) web tools with melting temperatures near or at 60°C, 40-60% GC content, and exon spanning amplicons between 100-200bp (Table S1). All miRNA forward primers were identical to mature miRNA sequences gathered at miRBase (7, 8) with reverse universal primers complementary to the proprietary universal adapter sequence at the end of cDNAs generated using qScript microRNA cDNA Synthesis kits (Quanta Biosciences). qRT-PCR was conducted on an ABI 7900HT thermocycler (Applied Biosystems) using SYBR Green Master Mix (Life Technologies) or the PerfeCTa SYBR Green SuperMix (Quanta Biosciences) per manufacturer protocols at 40 cycles, and data analyzed on SDS v.4.1 software (Applied Biosystems). Baselines for each gene were set at cycle 3 through to one cycle prior to logarithmic amplification and universal Ct thresholds set at approximately the lower quarter of the linear curve. HPRT and SCARNA17 primer assays were used as normalizing genes for large and small RNA derived cDNA respectively. The Ct calculation was used to estimate fold change from normalized Ct values between uninfected and infected sample groups (17).

Bioinformatic Analysis

Final high quality small RNA derived sequence reads, trimmed of 5' and 3' adapters, were aligned to all human mature miRNA sequences available through miRBase version 14 (7, 8) using megaBLAST (parameters: e-value 0.1, identity 85%, word size: 4, mismatch penalty: -4, match reward: 5, gap opening penalty: 8, gap extension penalty: 6, filtering: false). Read counts of identified mature miRNAs were normalized as counts per million reads (CPM); where: $CPM = (\text{number of sample miRNA reads} \div \text{total number of sample reads}) \times 10^6$. Despite that no evidence exists to indicate that *L. major* or *L. donovani* produce their own small interfering RNA transcripts (18, 19), any read or published miRNA sequence that displayed high similarity to any *Leishmania spp.* data published at EuPathDB

(20) were removed from downstream analysis. The miRanalyzer web tool (21) was used similarly to identify known mature miRNAs, but also isomiRs, tRNAs, repeat elements, and fragments of larger transcripts, as well as prediction of novel new miRNAs based on homology to hairpin structures in the human transcriptome. All transcript target predictions for found miRNAs were pulled from the microRNA Data Integration Portal (mirDIP) (22). mirDIP integrates multiple target prediction databases and provides a standardized score for each miRNA:transcript relationship. Only target predictions in the top third and top 1%, and predicted by at least two different algorithms, were collected for downstream analysis. Predicted target gene transcript lists for differentially expressed microRNAs were used to identify enriched KEGG Pathways by hypergeometric tests with Benjamini-Hochberg adjustments with the Homo sapiens genome as a reference set using the WEB-based Gene Set Analysis Toolkit (WebGestalt) (23).

Statistical Analysis

All correlation and other statistical tests were performed using GraphPad Prism v.5 (GraphPad Software) and R base v.3.1.0.

RESULTS AND DISCUSSION

Our analysis used next generation sequencing to comprehensively examine the expression of mature miRNAs in human MDDCs and MDMPs infected with either *L. major* or *L. donovani* parasites—species responsible for highly divergent clinical pathologies. Following sequence identification and copy number normalization, 104 mature miRNAs were identified that were present in either uninfected DC or MP and in at least one species infection (in either cell type) and among a majority of donors. This methodology enabled assessment of differential miRNA expression between uninfected and infected cells, whereby substantive changes could be attributed to *Leishmania* infection (Figure 1A). The NGS expression profiles of five miRNAs found in all cell types and infection contexts were validated by qRT-PCR using the same donor samples (Figure 1B).

Eight hours post-infection was selected as the RNA isolation end point due to prior evidence that *L. major* infected human host cells exhibit significant up-regulation of the pro-inflammatory cytokine subunit IL-12p40 at this same time point, yet *L. donovani* infected cells express little to none (24, 25). Moreover, a dynamic differential expression profile of interferons occurs during early infection, with maximal IFN γ production occurring at eight hours (26). Therefore, this time point is likely a time at which critical host regulatory mechanisms of early responses to *Leishmania* are active. The most striking result observed was the general suppression of miRNAs in *L. major* infected DC and up-regulation in DC infected with *L. donovani* (Figure 1C). Indeed, 95 miRNAs were identified in *L. major* infected DC that had average expression levels greater than or equal to 1.2 (7.8% of total identified) or less than or equal to -1.2 (84.5%) fold difference compared to uninfected cells (Table S2). Among *L. donovani*-infected DC, only 40 mature miRNAs were identified; 52.5% of which were expressed greater than or equal to 1.2 on average, and 30% expressed less than or equal to -1.2 fold difference compared to uninfected cells. In the case of MP infections, the number of mature miRNAs identified, as well as the percentage of those greater than or equal to 1.2 or less than or equal to -1.2 average fold difference compared to

uninfected cells, were much more equivalent between MPs infected with *L. major* versus those infected with *L. donovani* (Table S2).

To date, few studies have examined the regulatory role of host cell miRNAs during *Leishmania* infections (27-29). These studies either focused attention on a select few miRNAs of interest as dictated by the limitations of microarrays, or extracted cells from infected murine model organisms. Comparing the findings presented herein to that of human MDMPs at 6 and 12 hours post-*L. major* infection (29), we identified only 26 of the same miRNAs that were consistently dis-regulated in the previous study. Moreover, only 9 miRNAs in our study were regulated in the same differential manner compared to uninfected MPs. We attribute this discrepancy to three main factors: 1) our expression data are derived from NGS methods as opposed to qRT-PCR microarray, where sequencing approaches do not have thermodynamic biases usually associated with qRT-PCR platforms (30, 31); 2) we utilized an acid-phenol:chloroform procedure for the extraction of total RNA from *in vitro* cell samples which has been shown to specifically isolate small RNAs at higher quality concentrations, increasing the reproducibility of NGS results, compared to RNA isolation by the Trizol method (32, 33); and 3) we infected cells with a different strain of *L. major* (MHOM/IL/80/Friedlin, compared to MHOM/TN/95/GLC94). It has been well established that even among the same species of *Leishmania*, different laboratory strains exhibit differential effects upon host cell molecular biology (34). Such differential effects may extend toward miRNA expression profiles.

Biological pathway enrichments were performed using all potential *in silico*-predicted targets of up- or down-regulated miRNAs (Table 1). The mitogen-activated protein kinase (MAPK) signaling pathway was significantly enriched among possible target genes regardless of relative expression directionality, cell type, or infecting *Leishmania* species. The influence of *Leishmania* upon MAPK signaling has long been established (35), and it comes as no surprise that these parasites would potentially influence miRNA expression related to many genes associated with that pathway. Similarly, the endocytosis pathway, necessary for parasite uptake and establishment of infection, was also enriched in all instances. However, Janus kinase-signal transducer and activator of transcription (JAK-STAT) and tumor growth factor beta (TGF- β) signaling pathways were among the top ten most significantly enriched KEGG pathways only for miRNAs up-regulated in cells infected with *L. donovani*. Suppressor of cytokine signaling 4 (SOCS4), a negative regulator of JAK-STAT signaling (36), is an *in silico* predicted and experimentally validated target of let-7a (37)—a miRNA that our NGS data showed to be selectively up-regulated in *L. donovani* infected DCs and MPs, but down-regulated in *L. major* infected cells (Figure 1A, Table S2). As well, miR-21 has also been proven by several investigators to target SMAD7 (38-43), the negative regulator of TGF- β signaling. These results suggest that any modulations to these cellular pathways may be more heavily influenced by effects on host miRNAs during *L. donovani* infections than during *L. major* infections.

To explore whether biological evidence exists for *in silico* miRNA target predictions, qRT-PCR was utilized to examine the expression of three gene transcripts that are associated with TGF- β signaling: SMAD7, PU.1, and TRAF6. These genes were predicted to be targets of miR-21, miR-155, and miR-146b-5p, respectively. Concentrating on DC expression

specifically, miR-21 was inversely correlated with SMAD7 expression ($p = 0.005$, Pearson correlation coefficient = -0.995), only in DC infected with *L. donovani* (Figure 1D). Conversely, miR-146b-5p was positively correlated with TRAF6 ($p = 0.01$, $r = 0.95$). Although miR-146b-5p is known to directly target TRAF6 (44-47), the positive correlation of these factors in *L. donovani*-infected DC may be explained in that miR-146b-5p was found to be part of negative feedback loop of TGF- β signaling, where the miRNA's expression was promoted by TGF- β signaling as a means of pathway regulation (48). So, while there may be a positive correlation, the relationship may be based more on a higher level of expression activation rather than an actually miRNA:target association. While *L. major* infected DCs display a similar trend to that of these two gene transcripts, no significant correlations were observed. Critical pathway target relationships such as these, point to possible selective effects of *Leishmania* species on host cell immunological signaling. The transcription factor PU.1 (SPI1) was positively correlated with miR-155 ($p = 0.038$, $r = 0.962$) in *L. major* infected DCs only (Figure 1E). However, this same transcription factor has been shown to be both an activator (49) and a target (50) of miR-155, and is not exclusively specific to the TGF- β pathway alone. We also tested the expression of let-7a and its JAK-STAT pathway target, SOCS4, but found no significant correlations in DCs infected with either parasite species (data not shown). Not only does each *Leishmania* species influence broad differential miRNA expression profiles, we show that these differences are correlated with changes in putative target transcript presence as well.

In comparing mature miRNA expression profiles between the same host cell types infected by either *L. major* or *L. donovani*, several variations were observed, which have generated additional hypotheses regarding host regulatory disruption during infection (Figure 1A, Table S2). Among miRNAs identified both in cells infected with *L. major* and *L. donovani*, those infected with *L. donovani* parasites, regardless of cell type, exhibited higher expression of miRNAs compared with *L. major* infected cells. Members of the let-7 family of miRNAs are well represented in the dataset. Most apparent are the dichotomous expression patterns of let-7a and let-7b, where only DCs and MPs infected with *L. donovani* display up-regulation of those miRNAs and cells infected with *L. major* were observed to down-regulate the same miRNAs. The let-7 family has been shown to be involved in targeting transcripts of genes responsible for the induction of inflammation during bacterial infections as possible feedback inhibitors (51). Given that *L. major*, and not *L. donovani*, has been shown in human host cells to promote the production of pro-inflammatory cytokines, such as IL-12 (24, 34), it is possible that these opposing expression profiles for let-7a and let-7b are related to the species-specific mechanisms of inflammation. MiR-103 was expressed in a similar pattern to that of let-7a/b, where both cell types infected with *L. donovani* up-regulated the miRNA compared with the down-regulation observed in *L. major* infected cells. Insulin-like Growth Factor 1 (IGF-1) has been shown to be associated with *Leishmania* parasite infection and survival (52, 53), yet this same gene contributes to the down-regulation of miR-103 expression, at least among intestinal crypt cells (54). The differential expression of miR-103 among cells infected with different species of *Leishmania* may be a factor of IGF-1 expression mediated by interferon regulation (52). Additionally, one of the most up-regulated miRNAs identified was miR-511 (FC = 9.57),

but only in DCs infected with *L. donovani*. This miRNA has been implicated in the positive activation of toll-like receptor 4 (TLR4) in MDSCs and MDMPs (55). TLR4 up-regulation was identified as a part of the global immune response to *Leishmania* infections (56), which both *L. major* and *L. donovani* have been shown to impair in macrophages (57, 58) in order to avoid immune destruction. Leishmanial impairment of TLR4 might also work at the miRNA level through selective inhibition of miR-511 in MPs, but not DCs based on this evidence.

We have provided evidence for differential influences of *Leishmania* species on total mature microRNA expression patterns during early infection. Coupled with examination of predicted target gene transcript expression, our results demonstrate that the presence of variable types and amounts of miRNAs may impact host immunological responses in a species and cell type specific manner. Given that we have shown significant correlations between miR-21 and miR-146b-5p in *L. donovani* infected DCs and specific members of the TGF- β signaling pathway (SMAD7 and TRAF6), not found in *L. major* infected DCs, it is worthwhile to investigate this pathway further as it pertains to miRNA regulation. Additional examination of these phenomena are necessary, using multi-dimensional methods to concurrently assess temporal effects of infections on microRNA, target transcript, and protein product expressions. This information will assist in furthering our knowledge of parasitic influences upon host cell responses at a molecular level, ultimately providing opportunities to investigate new targets for rapid diagnostics or even therapeutic interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1. Expression profiles of mature human host cell miRNAs and predicted target gene transcripts during *Leishmania* infections

(A) Heatmap of all mature miRNAs identified by NGS in MDDCs and MDMPs infected with *L. major* or *L. donovani*. The color scale is based on average fold change difference in expression between infected versus uninfected paired donor cells of the same type (N = 4). Black squares are those microRNAs that were not found in a particular cell type or infection context. (B) Validation of average differential fold change expressions by qRT-PCR for five miRNAs identified with NGS in both infection contexts and cell types (Pearson correlation = 0.665, p-value = 0.002). (C) Log₂ ratio expression of *Leishmania*-infected host cell microRNAs over uninfected cells. Black lines represent mean ratios and hashed lines represent log₂ of ± 0.585 (fold change ± 1.5). (D, E) qRT-PCR assessment of three miRNA expressions in *L. donovani* and *L. major* infected MDDCs and three *in silico* predicted gene transcript targets from four matched human blood donors. Bars represent significant Pearson correlations (p < 0.05), with plus signs indicating a positive correlation and minus signs indicating negative correlations between miRNAs and predicted target gene transcripts.

TABLE 1

Pathway enrichments found among all *in silico* predicted gene transcript targets for up- or down-regulated mature miRNAs identified in *Leishmania* infected MDCCs and MDMPs by NGS.

KEGG Pathway	KEGG Entry No.	Predicted Targets of UP-regulated microRNAs				Predicted Targets of DOWN-regulated microRNAs			
		DC Ld	DC Lm	MP Ld	MP Lm	DC Ld	DC Lm	MP Ld	MP Lm
Metabolic pathways	1100	X	-	-	X	-	X	X	X
MAPK signaling pathway	4010	X	X	X	X	X	X	X	X
ErbB signaling pathway	4012	-	-	-	-	-	-	X	-
Calcium signaling pathway	4020	-	-	X	-	-	-	-	-
p53 signaling pathway	4115	-	-	-	-	-	-	-	X
Ubiquitin mediated proteolysis	4120	-	-	-	-	X	X	-	X
Protein processing in endoplasmic reticulum	4141	-	X	-	-	-	-	-	X
Endocytosis	4144	X	X	X	X	X	X	X	X
Wnt signaling pathway	4310	-	X	-	X	-	-	-	-
TGF-beta signaling pathway	4350	X	-	X	-	-	-	-	-
Axon guidance	4360	X	-	-	X	X	X	X	-
Focal adhesion	4510	X	X	X	X	X	X	X	X
Jak-STAT signaling pathway	4630	X	-	X	-	-	-	-	-
Neurotrophin signaling pathway	4722	X	X	X	X	X	X	X	-
Regulation of actin cytoskeleton	4810	X	X	X	X	-	X	X	X
Insulin signaling pathway	4910	-	-	-	X	X	X	X	-
GnRH signaling pathway	4912	-	X	-	-	-	-	-	-
Melanogenesis	4916	-	X	-	-	-	-	-	-
Pathways in cancer	5200	X	X	X	X	X	X	X	X
Renal cell carcinoma	5211	-	-	-	-	X	-	-	-
Prostate cancer	5215	-	-	-	-	X	-	-	X
Dilated cardiomyopathy	5414	-	-	X	-	-	-	-	-

Top 10 most significant KEGG pathways enriched among the lists of predicted microRNA target gene transcripts using the WebGestalt online tool (Benjamini & Hochberg adjusted p-values < 0.000001). Each pathway name and KEGG number are listed. "X" marks indicate a significant enrichment in a particular cell type and infection context. Up- or down-regulation of miRNAs is relative to uninfected cells of the same type. Select pathway names are emboldened for added emphasis.