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Analysis of the miRNA profile in C6/36 cells persistently infected with dengue virus type 2

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ABSTRACT

Dengue virus (DENV) is the most important arbovirus in the world; DENV is transmitted by the *Aedes* genus of mosquitoes and can establish a life-long persistent infection in mosquitoes. However, the exact mechanism by which persistent infection is established remains unknown. In this study the differential expression of miRNAs was analysed by deep sequencing and RT-qPCR using a previously established C6/36-HT cell line persistently infected with DENV 2 (C6-L) as a model. miR-927, miR-87, miR-210, miR-2a-3p, miR-190 and miR-970 were up-regulated, whereas miR-252, miR-263a-3p, miR-92b, miR-10-5p miR-9a-5p, miR-9a-1, miR-124, miR-286a and miR-286b were down-regulated in C6-L cells compared with C6/36 cells acutely infected with the same virus or mock-infected cells. Deep sequencing results were validated by RT-qPCR for the highly differentially expressed miR-927 and miR-9a-5p, which were up- and down-regulated, respectively, compared with both acutely and mock-infected C6/36 cells. The putative targets of these miRNAs include components of the ubiquitin conjugation pathway, vesicle-mediated transport, autophagy, and the JAK-STAT cascade as well as proteins with endopeptidase activity. Other putative targets include members of the Toll signalling pathway and proteins with kinase, ATPase, protease, scavenger receptor or Lectin C-type activity or that participate in fatty acid biosynthesis or oxidative stress. Our results suggest that several specific miRNAs help regulate the cellular functions that maintain equilibrium between viral replication and the antiviral response during persistent infection of mosquito cells. This study is the first report of a global miRNA profile in a mosquito cell line persistently infected with DENV.

1. Introduction

MicroRNAs (miRNAs or miRs) are small (approximately 20–25 nucleotides [nt] in length) highly conserved non-coding RNAs that participate in several processes in eukaryotic cells, including differentiation, homeostasis, metabolism, apoptosis, haematopoiesis and adaptive and innate immunity (Lu and Liston, 2009; Kim et al., 2009; Gangaraju and Lin, 2009; Kincaid and Sullivan, 2012). Initially, a miRNA is generated in its precursor form containing stem-loop hairpin structures with imperfect complementarity (pri-miRNA) from a primary transcript by RNA pol II in the cell nucleus. Then, the protein complex Drosha (RNase III endonuclease)/Pasha (DGCR8 in vertebrates) cleaves the pri-mRNA at the base of a hairpin stem to produce a ~70 nt pre-miRNA, which is subsequently transported to the cytoplasm by exportin 5 to be processed by the RNase III enzyme Dicer. The resulting 19- to 25-bp product contains a "guide" strand of RNA, which is incorporated into the Argonaute protein complex RISC (RNA-induced silencing complex) to promote recognition of the complementary target sequence, pri-

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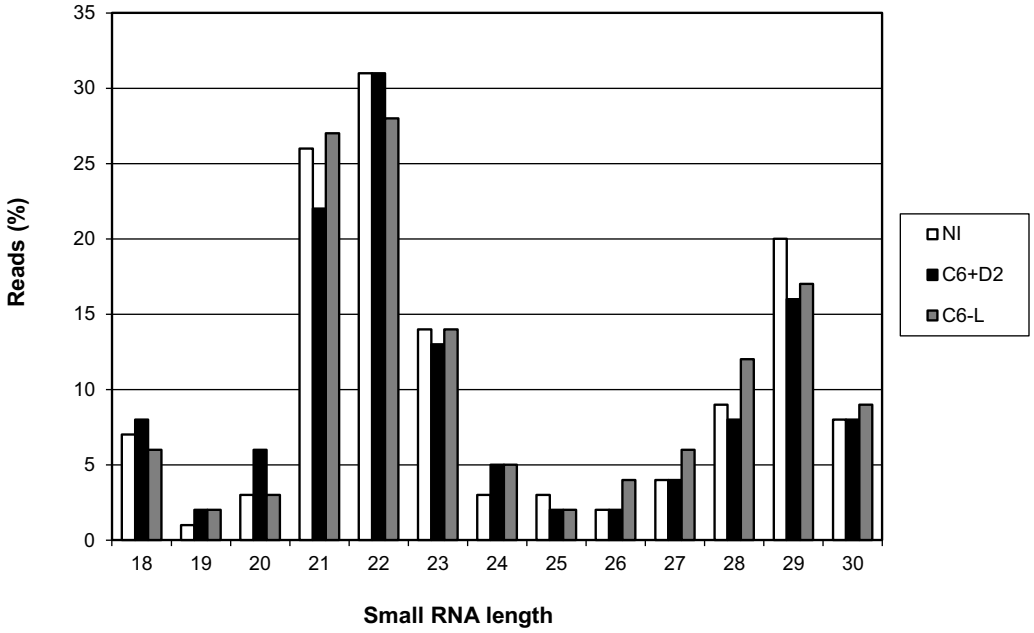

Fig. 1. Distribution of small RNAs by length. NI, mock-infected C6/36-HT cells; C6 + D2, C6/36-HT cells infected with DENV 2 for 48 h; C6-L, C6/36-HT cells persistently infected with DENV 2. The number of reads is indicated in percentage.

marily in the 3′ untranslated region (3′ UTR) but also in the 5′ UTR and the open reading frame of messenger RNAs (mRNAs), to induce mRNA degradation or silencing (Lu and Liston, 2009; Kim et al., 2009; Gangaraju and Lin, 2009; Kincaid and Sullivan, 2012; Swevers et al., 2013; Slonchak et al., 2014; Asgari, 2014). In addition to binding miRNAs, RISC is able to degrade double-stranded viral genomes by generating small interfering RNAs (siRNAs) (Lu and Liston, 2009). In mosquitoes, the Dicer-1/Argonaute-1 proteins are associated with miRNA biosynthesis, whereas Dicer-2/Argonaute 2 proteins are associated with siRNAs (Asgari, 2014; Hussain et al., 2012).

Flaviviruses are enveloped, single-stranded RNA viruses with positive polarity and are clinically important pathogens in humans (Roby et al., 2014). Dengue virus (DENV) is a flavivirus that causes fever, haemorrhagic fever, and fatality in more than 100 countries worldwide. There are four serotypes of DENV (1–4), which are all transmitted by mosquitoes of the *Aedes* (*Ae.*) genus (Malavige et al., 2004).

Although DENV replication has been detected in human red blood cells stored for transfusion after 35–42 days post-infection (Sutherland et al., 2016), the virus usually causes an acute infection characterised by massive viral replication and high viral load, which is usually resolved by the host's immune response. In contrast, flaviviruses can establish an apparent non-pathological persistent infection in the mosquito vector (Kanthong et al., 2010). Because insects do not have conventional immunological memory, non-pathological persistent infection is a convenient strategy for tolerating the presence of virus (Goic and Saleh, 2012; Blair and Olson, 2014). The underlying mechanisms of establishing and maintaining persistent infection are not completely understood but depend on several factors of both the virus and the cell (reviewed in Mlera et al., 2014 and Salas-Benito and De Nova-Ocampo, 2015).

miRNAs are key components of innate immunity during viral infection and are critical in virus-host interactions (Asgari, 2014). Several miRNAs participate in controlling infection with human immunodeficiency virus (HIV) (Huang et al., 2007), vesicular stomatitis virus (VSV) (Otsuka et al., 2007), hepatitis C virus (HCV) (Pedersen et al., 2007), and human cytomegalovirus (HCMV) (Wang et al., 2008), among others. Notably, recent studies have revealed that miRNAs play an important role during infection with flaviviruses, such as West Nile (WNV) (Slonchak et al., 2014; Skalsky et al., 2010) and DENV (Campbell et al., 2014; Hussain et al., 2013; Zhang et al., 2013; Yan et al., 2014; Liu et al., 2015) in mosquitoes (Campbell et al., 2014; Skalsky et al., 2010; Hussain et al., 2013; Zhang et al., 2013; Yan et al., 2014; Liu et al., 2015; Etebari et al., 2015) and in mosquito cell lines (Slonchak et al., 2014; Skalsky et al., 2010; Hussain et al., 2013; Zhang et al., 2013; Yan et al., 2014). Some results have suggested that miRNAs are involved in the persistent infection of flaviviruses in mosquitoes, but an exact role has yet to be defined (Campbell et al., 2014; Skalsky et al., 2010). Because miRNAs play an important role in regulating gene expression in several cellular pathways, we analysed the miRNA expression profile in a previously established C6/36-HT cell line persistently infected with DENV 2 after 57 week of infection using deep sequencing (Juárez-Martínez et al., 2013). In our knowledge, this is the first report about the global miRNA expression profile in a persistently infected mosquito cell line with DENV.

2. Material and methods

2.1. Cells and viruses

The C6/36-HT *Ae. albopictus* cell line (Igarashi, 1978), which is adapted to grow at 35 °C (Kuno and Oliver, 1989) (kindly donated by Dr. Goro Kuno), was cultured in 75-cm^2 flasks (Corning) in MEM (Gibco) supplemented with vitamins (Gibco), 10% foetal bovine serum, 0.034% sodium bicarbonate (J.T. Baker), and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin) (Sigma). C6/36-HT cells at 57 weeks of persistent infection with DENV 2 (C6-L cells) (Juárez-Martínez et al., 2013) were cultured in the same conditions. BHK-21 cells were grown at 37 °C in glass Petri dishes in MEM (Gibco) supplemented with 0.034% sodium bicarbonate (J.T. Baker), 10% foetal bovine serum, and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin) (Sigma).

The DENV 2 New Guinea C strain (NGC), donated by InDRE (Instituto Nacional de Diagnóstico y Referencia Epidemiológicos, Mexico City, Mexico), was propagated in newborn mice as described previously (Gould and Clegg, 1991). Aliquots were stored at −80 °C

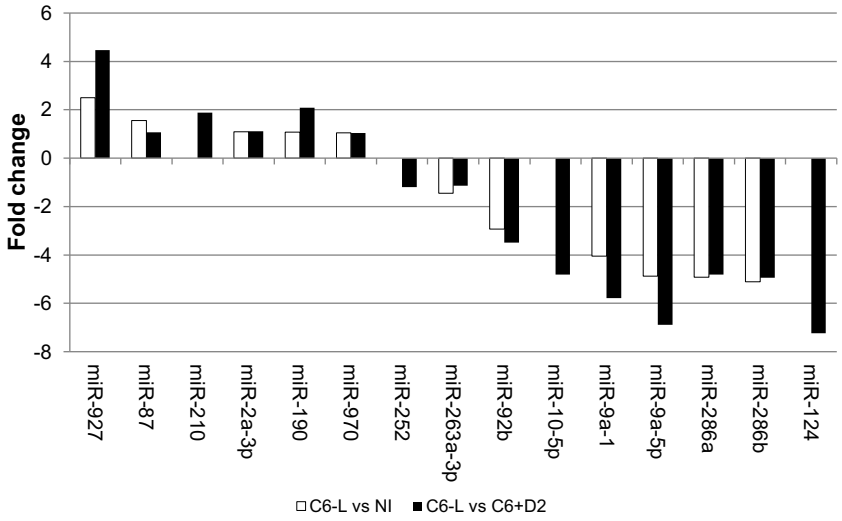

Fig. 2. Differential expression of miRNAs determined by edgeR software. Relative expression levels of miRNAs in C6-L vs mock-infected C6/36-HT cells are indicate in white bars and vs C6/36-HT cells acutely infected with DENV 2 in black bars. Only the miRNAs with a fold change higher than 1 and with statistical significance ($p \leq 0.05$) are shown.

Table 1
Total reads of miRNAs with a significant fold change in mock- and persistently infected C6/36-HT cells with DENV.

miRNA	Localization	Sequence	C6-L	NI	Total	logFC	*P*
aal-miR-252	JXUM01S002713: 109849–109950: −1	CUAAGUACUAGUGCCGCAGGAGU	14203	21130	35333	−0.8884	0.00111
aal-miR-970	JXUM01S003358: 118513–148689:1	UCAUAAGACACACGCGGCUAU	49604	9436	59040	1.0419	0.00013
aal-miR-92b	JXUM01S002680: 71276–71356:−1	AAUUGCACUUGUCCCGGCCUGC	1976	14957	16933	−2.9358	1.18E-23
aal-miR-2a-3p	JXUM01S000772: 379774–379840:−1	ACUCUCAAAGUGGUUGUGAAA	15530	4201	19730	1.0951	0.00049
aal-miR-190	JXUM01S003187: 116825–116915:−1	AGAUAUGUUUGAUAUUCUUGGUU	9036	3554	12590	1.0714	0.00010
aal-miR-87	JXUM01S000831: 164893–187790:1	GUGAGCAAAUUUUCAGGUGUGU	2854	405	3259	1.5543	8.06E-08
aal-miR-286a	JXUM01S004357: 137645–137745:1	UGACUAGACCGAACACUCGCGUCC	84	1137	1221	−4.9155	2.02E-37
aal-miR-286b	JXUM01S004357: 137645–137745:1	UGACUAGACCGAACACUCGUAUCCC	74	1145	1219	−5.1104	7.87E-39
aal-miR-9a-1	JXUM01S002683:138264–138325:1	UCUUUGGUUAUCUAGCUGUAUGAGU	14	103	117	−4.0545	7.16E-12
aal-miR-927	JXUM01S002252: 80522–80601:1	CAAAGCGUUUGGGUUCUGAAAC	86	6	92	2.4996	7.92E-05
aal-miR-210	JXUM01S000208: 491882–530099:1	CUUGUGCGUGUGACAACGG	20	3	23	1.4598	0.1665
aal-miR-263a-3p	JXUM01S002713: 104096–111534:−1	CUAAGUACUAGUGCCGCAGGAG	28	31	59	−1.4527	0.00152
aal-miR-9a-5p	JXUM01S002683:138264–138325:1	UCUUUGGUUAUCUAGCUGUAUGA	6	158	164	−4.8712	7.48E-14
aal-miR-10-5p	JXUM01S002702: 100165–101063:1	ACCCGUAGAUCCGAACUUGUU	8	18	26	−1.3895	0.12453

NI, mock-infected C6/36 cells; C6-L, C6/36 cells persistently infected with DENV 2. All values represent the average of lectures performed in duplicate.

(Sanyo, Vip Series MDF-U53VC). The virus titre was determined by plaque assay using BHK-21 cell monolayers grown in 24-well plates as previously reported (Juárez-Martínez et al., 2013).

2.2. Acute DENV 2 infection

C6/36 cell monolayers at 80% confluence were infected with DENV 2 at an MOI of 1 for 2 h at 37 °C with gentle shaking. Then, the monolayers were washed three times with 0.5% PBS-SFB, and fresh medium was added. The infection was allowed to proceed for 48 h at 35 °C and was confirmed by plaque assay and RT-PCR (see below). The acutely infected cells were used as a positive control. Mock-infected C6/36 cells were used as a negative control.

2.3. RNA isolation and deep sequencing

Total RNA and small RNAs were isolated from C6-L cells and from mock-infected or acutely infected C6/36-HT cells using a mirVana Kit (Ambion) according to the manufacturerís instructions. RNA concentration and purity were determined by measuring the absorbance at 260 nm (A_{260}) and the $A_{260/280}$ ratio, respectively, using a microplate spectrophotometer (Epoch, Biotek). To confirm the presence of DENV 2 in C6-L and C3/36 cells, total RNA was subjected to RT-PCR using a MyTaq one-step RT-PCR kit (BioLine) and specific primers for the NS4A-NS4B-NS5 region of the DENV genome (primers DV2M15 and DV2M16) as previously reported (Juárez-Martínez et al., 2013). *Aedes aegypti* S7 rRNA was amplified as an endogenous control (forward primer: 5′-GGGACAAATCGGCCAGGCTATC-3′; reverse primer: 5′-TCGTGGACGCTTCTGCTTGTTG-3′).

The integrity of small RNAs was assessed through 15% denaturing polyacrylamide–8 M urea gel electrophoresis. The concentration and purity were confirmed in an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Two biological samples for each condition were sequenced at UUSMD (Unidad Universitaria de Secuenciacion Masiva de DNA; UNAM, Cuernavaca, Mexico). Briefly, adapters (5′ and 3′) were ligated to small RNAs according to the manufacturerís instructions, and the products were converted to cDNA by RT and then amplified by 15 PCR cycles to produce cDNA-tag libraries. The PCR products were sequenced using an Illumina Genome Analyzer GAllx (Illumina, San, Diego, CA, USA). Sequence data is available at Sequence Read Archive (SRA) from National Center of Biotechnology Information (NCBI), accession number SRP099461.

2.4. Bioinformatics

Tag files of each sample were processed to remove adapters and low-quality sequences, and sequences between 18 and 30 nt in length were selected for future analysis. Sequence information for the *Ae. albopictus* and *Ae. aegypti* genomes was obtained from VectorBase (http://www.vectorbase.org,

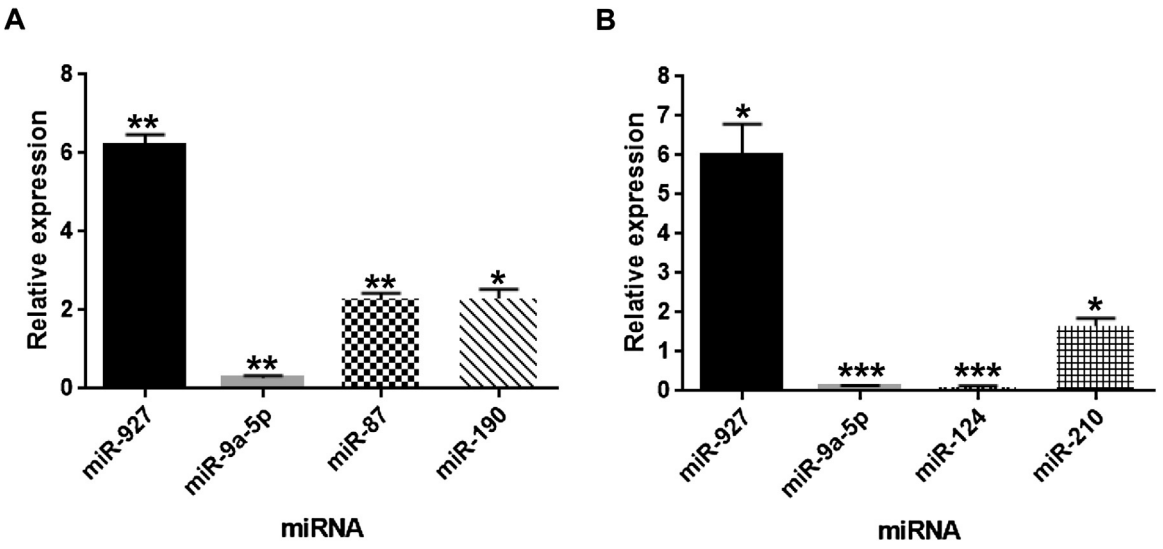

Fig. 3. Validation of the expression of miRNAs by RT-qPCR. A, Mock-infected (NI) vs C6-L cells. B, C6/36-HT cells acutely infected with DENV 2 vs C6-L cells. Relative expression determined by $2^{-\Delta\Delta Ct}$ formula. A representative experiment performed in triplicate is shown. Asterisks indicate statistical significance; *p < 0.05, **p < 0.005 ***p < 0.0001.

Table 2
Total reads of miRNAs with a significant fold change in acutely and persistently infected C6/36-HT cells with DENV.

miRNA	Localization	Sequence	C6-L	C6 + D2	Total	logFC	*P*
aal-miR-252	JXUM01S002713: 109849–109950: −1	CUAAGUACUAGUGCCGCAGGAGU	14203	38204	52407	−1.2027	2.11E-05
aal-miR-970	JXUM01S003358: 118513–148689:1	UCAUAAGACACACGCGGCUAU	49604	13088	62692	1.0376	0.00023
aal-miR-92b	JXUM01S002680: 71276–71356:−1	AAUUGCACUUGUCCCGGCCUGC	1976	34459	36435	−3.4850	1.54E-29
aal-miR-2a-3p	JXUM01S000772: 379774–379840:−1	ACUCUCAAAGUGGUUGUGAAA	15530	6266	21796	1.1084	8.90E-05
aal-miR-190	JXUM01S003187: 116825–116915:−1	AGAUAUGUUUGAUAUUCUUGGUU	9036	2264	11300	2.0834	1.62E-34
aal-miR-87	JXUM01S000831: 164893–187790:1	GUGAGCAAAUUUUCAGGUGUGU	2854	777	3631	1.0708	0.00034
aal-miR-286a	JXUM01S004357: 137645–137745:1	UGACUAGACCGAACACUCGCGUCC	84	1327	1411	−4.8178	1.60E-34
aal-miR-286b	JXUM01S004357: 137645–137745:1	UGACUAGACCGAACACUCGUAUCCC	74	1329	1403	−4.9489	3.80E-35
aal-miR-9a-1	JXUM01S002683:138264–138325:1	UCUUUGGUUAUCUAGCUGUAUGAGU	14	406	420	−5.7809	1.47E-27
aal-miR-927	JXUM01S002252: 80522–80601:1	CAAAGCGUUUGGGUUCUGAAAC	86	2	88	4.3611	6.34E-07
aal-miR-124	JXUM01S004796: 56100–120643:1	UAAGGCACGCGGUGAAUGC	1	74	75	−7.2298	1.68E-08
aal-miR-210	JXUM01S000208: 491882–530099:1	CUUGUGCGUGUGACAACGG	20	3	23	1.8771	0.05
aal-miR-263a-3p	JXUM01S002713: 104096–111534:−1	CUAAGUACUAGUGCCGCAGGAG	28	32	60	−1.1412	0.00660
aal-miR-9a-5p	JXUM01S002683:138264–138325:1	UCUUUGGUUAUCUAGCUGUAUGA	6	762	768	−6.8916	1.27E-32
aal-miR-10-5p	JXUM01S002702: 100165–101063:1	ACCCGUAGAUCCGAACUUGUU	8	218	226	−4.8145	1.49E-16

C6 + D2, C6/36 cells infected with DENV 2 for 48 h; C6-L, C6/36 cells persistently infected with DENV 2. All values represent the average of lectures performed in duplicate.

Aedes-albopictus-Foshan_CONTIGS_ AaloF1.1 and Aedes-aegypti-Liverpool_CONTIGS_AaegL3), and the experimentally obtained sequences were mapped against each reference genome using the BWA program (Li and Durbin, 2009). Reads corresponding to rRNA, tRNA, snRNA, snoRNA, repeat, exon, and intron sequences were excluded. To compare miRNA abundance in both libraries for each condition, putative miRNA sequences were normalised using the protocol reported previously (Shrinet et al., 2014; Liu et al., 2015) expressed in tag per million of total reads (TPM: The count of the miRNA in a particular sample/Total miRNA counts from this sample × 1 million). To exclude miRNAs expressed at very low levels above background, sequences with a TPM of less than 10 were eliminated. Because *Ae. albopictus* miRNAs are not available in miRBase (http//mirbase.org), the sequences were mapped against known *Ae. aegypti* miRNA precursors and only perfectly matching sequences were considered as candidate miRNA genes (Liu et al., 2015).

To determine the effects of DENV 2 infection on cellular miRNA expression, miRNA profiles were compared between persistently infected C6-L cells and mock-infected and acutely infected C6/36 cells using edgeR software (Bioconductor, R). EdgeR is designed specifically for assessing data from biological replicates and uses a negative binomial distribution and empirical Bayes strategy to calculate changes in relative gene expression levels (Robinson et al., 2010). P-values < 0.05 were considered significant. miRNAs with a fold change higher than 1 were considered differentially expressed.

2.5. Target prediction of miRNAs and functional analysis

Target prediction was performed with Microcosm Target Version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/#) and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) using transcript information of *Ae. albopictus* in VectorBase (*Aedes-albopictus*-NIAID_GAPW01, and *Aedes-albopictus*-Foshan_Transcripts_ AaloF1.1). Only miRNAs with a p-value < 0.05 in the differential miRNA expression analysis were considered. Targets were selected according to the following program specifications: minimum free energy <−20 kcal/mol and p-value < 0.05. Gen Ontology (GO) terms were matched to the top five targets for functional annotation.

2.6. RT-qPCR

RT-qPCR was performed to confirm the differential expression of selected miRNAs using the TaqMan small RNA assay (Applied Biosystems) and specific TaqMan primers according to the manufacturer's instructions. 5.8S rRNA was used as a normalisation control for each condition. All reactions were performed in two

Table 3
Putative targets of differentially expressed miRNAs.

GO annotation	Protein	mfe	P-value	miRNA
Ubl conjugation pathway	E3 ubiquitin-protein ligase	−24.0 kcal/mol	1.000000E + 00	miR-927
	Small ubiquitin-related modifer	−23.5 kcal/mol	1.000000E + 00	
Proteolysis	Putative 26 s proteasome regulatory complex subunit	−27.8 kcal/mol	1.000000E + 00	miR-9a-1
Serine-type endopeptidase activity	Putative CLIP-domain	−23.5 kcal/mol	1.000000E + 00	miR-190
	serine protease	−22.3 kcal/mol	1.000000E + 00	miR-210
	Mannan-binding lectin serine protease 2	−20.8 kcal/mol	1.000000E + 00	miR-10-5p
	ATP-dependent Clp protease proteolytic subunit	−21.1 kcal/mol	1.000000E + 00	miR-2a-3p
Serine-type endopeptidase inhibitor activity	Putative serine-type endopeptidase	−26.8 kcal/mol	1.000000E + 00	miR-9a-5p
	inhibitor	−30.3 kcal/mol	1.000000E + 00	miR-263a-3p
		−28.0 kcal/mol	1.000000E + 00	miR-9a-1
Innate immune response	Peptidoglycan-recognition protein	−21.6 kcal/mol	1.000000E + 00	miR-190
JAK-STAT cascade	Putative suppressors of cytokine signaling	−21.1 kcal/mol	1.000000E + 00	miR-9a-5p
Scavenger receptor activity	Putative scavenger receptor	−22.1 kcal/mol	1.000000E + 00	miR-87
	Putative chorion	−23.2 kcal/mol	1.000000E + 00	miR-210
Integral component of membrane	Putative Toll-like	−27.0 kcal/mol	1.000000E + 00	miR-87
	receptor 5b	−24.0 kcal/mol	1.000000E + 00	miR-190
	Putative low-density lipoprotein receptor	−22.3 kcal/mol	1.000000E + 00	miR-124
Lysozyme activity	Lysozyme C-4	−22.8 kcal/mol	1.000000E + 00	miR-210
Peptidase activity	Putative furin-like protease 2	−22.7 kcal/mol	1.000000E + 00	miR-210
Response to oxidative stress	Glutathione peroxidase	−25.6 kcal/mol	1.000000E + 00	miR-263a-3p
Carbohydrate binding	Galectin	−24.3 kcal/mol	1.000000E + 00	miR-2a-3p
	Putative mannose lectin ergic-53 involved in glycoprotein traffic	−21.8 kcal/mol	1.000000E + 00	miR-87
Calcium-dependent phospholipid binding	Annexin	−21.2 kcal/mol	1.000000E + 00	miR-190
Calcium ion binding	Putative cad74a	−25.8 kcal/mol	1.000000E + 00	miR-9a-1
Protein kinase activity	Putative Ca2+/calmodulin-dependent protein	−24.3 kcal/mol	1.000000E + 00	miR-970
Structural constituent of cytoskeleton	Putative actin-binding cytoskeleton protein filamin	−23.6 kcal/mol	1.000000E + 00	miR-286a
	Putative Ca2+-binding actin-bundling protein	−23.8 kcal/mol	1.000000E + 00	miR-970
Carboxylic ester hydrolase activity	Putative lipase	−26.2 kcal/mol	1.000000E + 00	miR-2a-3p
		−26.8 kcal/mol	1.000000E + 00	miR-286a
	Carboxylic ester hydrolase	−26.9 kcal/mol	1.000000E + 00	miR-2a-3p
Phospholipid-translocating ATPase activity	Phospholipid-transporting ATPase	−22.8 kcal/mol	1.000000E + 00	miR-124
Fatty acid biosynthetic process	Elongation of very long chain fatty acids protein	−22.3 kcal/mol	1.000000E + 00	miR-10-5p
Translation initiation factor activity	Putative translation initiation factor 3 subunit a eIF-3α	−23.4 kcal/mol	1.000000E + 00	miR-124
Vesicle-mediated transport	Putative snare protein ykt6 synaptobrevin/vamp syperfamily	−26.6 kcal/mol	1.000000E + 00	miR-263a-3p
	Putative clathrin-associated protein medium chain	−25.5 kcal/mol	1.000000E + 00	miR-927
	Putative snare protein syntaxin 1	−27.3 kcal/mol	1.000000E + 00	miR-9a-1
ER to Golgi vesicle-mediated transport	Putative vesicle coat complex COPII subunit sec24/subunit sfb2	−22.8 kcal/mol	1.000000E + 00	miR-286a

Table 3 (*Continued*)

GO annotation	Protein	mfe	P-value	miRNA
	Putative vesicle coat complex COPII subunit sfb3	−22.9 kcal/mol	1.000000E + 00	miR-9a-5p
	Golgi SNAP receptor complex member 1	−25.8 kcal/mol	1.000000E + 00	miR-92b
Intracellular protein transport	SNAPIN protein homolog	−25.2 kcal/mol	1.000000E + 00	miR-92b
Protein transporter activity	Vacuolar protein sorting-associated protein 29	−23.2 kcal/mol	1.000000E + 00	miR-10-5p
	Putative cargo transport protein	−24.3 kcal/mol	1.000000E + 00	miR-92b
	Importin subunit alpha	−23.4 kcal/mol	1.000000E + 00	miR-10-5p
Regulation of exocytosis	Putative snare protein syntaxin 1	−26.6 kcal/mol	1.000000E + 00	miR-92b
Autophagy	Putative microtubule-associated anchor protein involved in autophagy and membrane trafficking	−20.4 kcal/mol	1.000000E + 00	miR-927
Cysteine-type peptidase activity	Putative cathepsin l	−31.8 kcal/mol	1.000000E + 00	miR-263a-3p
Regulation of pH	Sodium/hydrogen exchanger	−25.9 kcal/mol	1.000000E + 00	miR-970

biological samples in triplicate using an Mx3000p qPCR System (Agilent Technologies). The relative expression of each miRNA was determined by the $2^{-\Delta\Delta Ct}$ method. The data were analysed by GraphPad Prism software (GraphPad, San Ciego, CA, USA) using paired Student's *t*-test for statistical analysis. $P < 0.05$ was considered significant.

3. Results

3.1. Results of deep sequencing

The C6/36 cell line (from *Ae. albopictus*) has been used extensively to study persistent infection with certain flaviviruses, including DENV, Japanese encephalitis virus (JEV) and *Ae. albopictus* densovirus (Aal DNV) (Igarashi, 1979; Chen et al., 1994; Tsai et al., 2007; Kanthong et al., 2008; Kanthong et al., 2010). Although this cell line is deficient in Dicer 2, an RNase involved in the biogenesis of endo- and exo-siRNAs (Scott et al., 2010; Brackney et al., 2010; Morazzani et al., 2012; Blair and Olson, 2015), the activity of Dicer 1, which is responsible for the synthesis of miRNAs (Hess et al., 2011), is apparently normal because several miRNAs have been detected in C6/36 cells in response to WNV (Slonchak et al., 2014) and DENV infections (Yan et al., 2014).

According to a previous study (Igarashi, 1979), we established a C6/36-HT cell line persistently infected with DENV 2, designated as C6-L. After 42 weeks of infection in this cell line, the viral yield seem to be too low because infectious viral particles are no longer detected by plaque assay in BHK-21 cells, and no cytopathic effect (CPE) was observed. Furthermore, defective viral genomes were detected in these cells in addition to the wild type as well as some viral proteins such as NS1, E and NS5 (Juárez-Martínez et al., 2013). Since C6/36-HT cells are adapted to grow at 35 °C, all the experiments were performed at that temperature.

To define the miRNAs involved in persistent DENV 2 infection, we compared miRNA profiles of C6-L cells and acutely infected C6/36-HT cells, as well as mock-infected C6/36-HT cells. Total and small RNA populations were isolated using a mirVana Kit (Ambion). The quality of small RNAs was confirmed through 15% polyacrylamide-urea gel electrophoresis (Supplementary Fig. S1A). RT-PCR results confirmed the presence of the DENV 2 genome in the C6-L cells and in the acutely infected C6/36-HT cells (Supplementary Fig. S1B). As reported previously (Juárez-Martínez et al., 2013), a plaque assay performed in BHK-21 cells only detected infectious DENV 2 viral particles in the acutely infected C6/36-HT cells but not in the C6-L cells (data not shown).

Duplicates of small RNA samples obtained from mock-infected C6/36-HT cells, acutely infected C6/36 cells and C6-L cells were used for deep sequencing. After the adapters were removed, the total reads obtained were 13,934,811; 18,538,225; and 18,470,020 from mock-infected cells, C6/36-HT cells acutely infected with DENV 2, and C6-L, respectively (data not shown), which were similar results to a previous report (Liu et al., 2015). After sequences shorter than 18 nt and longer than 30 nt were eliminated, we observed that most of the remaining sequences (approximately 25–30%) correspond to RNAs of 21–22 nt in length in the three conditions tested (Fig. 1). These sequence lengths are in agreement with those of miRNAs, strongly suggesting that these sequences correspond to miRNAs. A second population of sequences was found (from 15 to 20%) corresponding to RNAs 29 nt in length (Fig. 1).

After all of these sequences were mapped using the *Ae. albopictus* (Chen et al., 2016) database (https://www.vectorbase.org), the number of reads was reduced to 4,389,481 sequences in mock-infected cells, 5,866,778 sequences in acutely infected C6/36-HT cells, and 6,371,532 sequences in C6-L cells. All sequences corresponding to tRNA, rRNA and snoRNAs were then eliminated, and the resulting profile of miRNA expression was normalised as described in the experimental section. Finally, 37 miRNAs were identified using the miRBase database (http://mirbase.org): 36 were shared in the 3 conditions tested, and only one, miR-124, was found in only the C6-L cells and acutely infected C6/36-HT cells (see supplementary Fig. S2). Of the 37 miRNAs, only 15 had a significant differential expression ($p < 0.05$) and therefore were relevant for this study (Fig. 2).

3.2. Differential expression of miRNAs

To characterise the miRNA profile during persistent infection with DENV 2, miRNA differential expression was analysed using edgeR software (Robinson et al., 2010; Samnath and Nettleton, 2014). Comparison of the miRNA expression profile in C6-L cells versus mock-infected C6/36 cells showed that five miRNAs (miR-927, miR-87, miR-2a-3p, miR-190, and miR-970) were up-regulated in persistently infected cells, and six (miR-263a-3p, miR92b, miR-9a-1, miR-9a-5p, and miR-286a and b) were

down-regulated (Table 1 and Fig. 2). In contrast, nine miRNAs (miR-252, miR-263a-3p, miR-92b, miR-10-5p, miR-9a-1, miR-9a-5p, miR-286a, miR-286b, and miR-124) were down-regulated and six (miR-927, miR-87, miR-210, miR-2a-3p, miR-190, and miR-970) were up-regulated in persistently infected versus acutely infected cells (Table 2 and Fig. 2). Interestingly, some miRNAs were up-regulated in C6-L cells compared with both control conditions (miR-927, miR-87, miR-2a-3p, miR-190, and miR-970) or down-regulated (miR-263a-3p, miR-92b, miR-9a-1, miR-9a-5p, miR-286a, and miR-286b), but others were altered only compared with acutely infected C6/36-HT cells. For example, miR-210 was up-regulated and miR-252, miR-10-5p and miR-124 were down-regulated in C6-L cells.

Six miRNAs with the highest significant differences according to the results of differential expression obtained with edgeR software were selected for validation by RT real-time PCR using specific TaqMan probes. The results confirmed that miR-927 and miR-9a-5p were significantly up-regulated and down-regulated respectively, in C6-L cells compared with mock-infected (Fig. 3A) and acutely infected (Fig. 3B) C6/36-HT cells.

The over-expression of miR-190 and −87 could be validated in C6-L cells compared with mock-infected cells (Fig. 3A) but not in C6/36-HT cells acutely infected with DENV 2 in which the difference was not statistically significant. The down-regulation of miR-124 and over-expression of miR-210 in C6-L cells compared with acutely infected cells were also corroborated (Fig. 3B).

3.3. Predicted miRNA targets

The transcriptome targets for each miRNA detected with significant differential expression were predicted using RNAhybrid and Micfrocosm target software and *Ae. albopictus* transcriptome information. The five most important targets for each miRNA were selected for further analysis, and GO annotation was determined (Table 3).

Proteins involved in ubiquitination, the innate immune response, the response to oxidative stress, cytoskeleton structure, fatty acid biosynthesis, intracellular protein transport, exocytosis regulation, autophagy and pH regulation were found. Additionally, proteins with serine or cysteine peptidase, carbohydrate-binding, calcium-dependent phospholipid-binding, kinase, carboxylic ester hydrolase, phospholipid-translocating ATPase, and translation initiation activity were identified (Table 3).

4. Discussion

4.1. Identification of relevant miRNAs in C6-L cells

A persistent viral infection is defined as a long-term infection where the antiviral response of the host and the replicative ability of the virus are in equilibrium resulting in a non-pathological infection (Blair et al., 2000). This type of infection is particularly important in flaviviruses because facilitates the vector to vertebrate transmission and it is an interesting model to study the virus-host interactions (Goic and Saleh, 2012). Mosquito cell lines have been extensively used to study persistent flavivirus infections because they are easy to manage, several variables can be controlled and the results are easy to interpret (Salas and De Nova 2015). Among them, C6/36 cells have been the most used cell line to study flavivirus persistence (Igarashi, 1979; Chen et al., 1994; Tsai et al., 2007; Kanthong et al., 2008; Kanthong et al., 2010). However the findings obtained in cell lines should be validate in the context of mosquitoes. We previously developed a C6/36-HT cell line persistently infected with DENV 2 denominated C6-L (Juárez-Martínez et al., 2013). Even this particular cell line is cultured at a temperature 7 °C warmer than the regular C6/36 cells, all experiments were performed at the same temperature. Additionally, C6/36-HT cells have been used previously to study several aspects of the replicative cycle of DENV such as virus-receptor interactions (Salas-Benito and del Angel, 1997; Salas-Benito et al., 2007; Vega-Almeida et al., 2013) and virus entry (Mosso et al., 2008) as well as cell stress response (González-Calixto et al., 2015). During the first 42 weeks of infection, C6-L cells generated infectious viral particles that were clearly detected by plaque assay in BHK-21 cells with variations in the size of plaques as reported previously (Randolph and Hardy, 1988; Igarashi, 1979; Chen et al., 1994). After that, the viral particles are no longer detected by this method indicating an important reduction in virus yield. However, the infection could be evidenced by the detection of the viral genome and proteins such as NS1, E and NS5 (Juárez-Martínez et al., 2013). All these results indicate an important modification in the virus-host interaction but the mechanisms involved are not well understood.

miRNAs, piwi-interacting RNAs (piRNAs) and siRNAs are the major small RNA regulatory elements present in mosquitoes, and their sizes are distinctive: 21–22 nt for siRNAs and miRNAs and 24–30 nt for piRNAs. miRNAs and siRNAs are generated in a Dicer-1- or Dicer-2-dependent manner, respectively, and piRNAs in a pathway that is Dicer-independent (Ghildiyal and Zamore, 2009; Hess et al., 2011; Léger et al., 2013). Several studies have demonstrated the importance of miRNAs in mosquitoes or mosquito cell lines during viral infection (Slonchak et al., 2014; Campbell et al., 2014; Skalsky et al., 2010; Hussain et al., 2013; Zhang et al., 2013; Yan et al., 2014; Liu et al., 2015); however, the exact role of these miRNAs during persistent infection has not been studied in detail. Because miRNAs play an important role in regulating the expression of genes affecting several cellular pathways, we analysed the miRNA expression profile by deep sequencing using a C6/36-HT cell line persistently infected with DENV 2 (C6-L) that was previously established and characterised in our laboratory (Juárez-Martínez et al., 2013) as a model of viral persistence.

Deep sequencing of small RNAs allowed to us to identify two main populations of 21–22 nt and 29 nt in length. Previous experiments performed in *Ae. albopictus* mosquitoes infected with DENV 2 have also identified these two populations of small RNAs. The 21–22 nt RNAs were detected in higher concentrations in non-infected mosquitoes, while the 27–28 nt RNAs were detected in higher concentrations in infected ones (Liu et al., 2015). These differences were not detected in our study, which could be related to the fact that we used a mosquito cell line and not mosquitoes. Regarding the 21–22 nt small RNA population, small viral interfering RNAs (vsiRNA) are 21 nt in length and are generated from double-strand viral RNA (secondary structures or viral replication intermediates) in a Dicer-2-dependent manner (Vodovar et al., 2012; Léger et al., 2013; Miesen et al., 2016). Because this population is less abundant in C6/36 cells infected with DENV 2 (Scott et al., 2010; Miesen et al., 2016) or Chikungunya virus (CHIKV) (Morazzani et al., 2012) than in Dicer-2 competent cells, we focused our study on miRNAs rather than vsiRNAs.

Curiously, minimal regulation of miRNAs has been observed in Aag2 acutely infected with DENV 2 (Miesen et al., 2016). However, our results indicate, like other previously reported, that miRNAs are regulated during DENV infection in a mosquito cell line (Yan et al., 2014). These conflicting data may be mainly associated with the cell type used, the multiplicity of infection, and the time of infection (acute vs persistent).

On the other hand, a population of small RNAs 25–29 nt in length that correspond to piRNAs has been detected in C6/36 cells infected with DENV 2 (Scott et al., 2010), Sindbis virus (SINV), La Crosse virus (LACV) (Vodovar et al., 2012; Miesen et al., 2015), CHIKV (Morazzani et al., 2012) or Rift Valley fever virus (RVFV) (Léger et al., 2013). piRNAs are small RNAs that contain a 2′-*O*methyl group

at their 3′ end and regulate transcription and the transposition of transposable elements. The piRNA precursors are transcribed by RNA Pol II from transposon sequences, and the primary piRNAs are then amplified by a mechanism denoted "ping-pong". piRNAs are generated in a Dicer-independent manner with the participation of Piwi (1–7 in *Ae. aegypti*), Aubergine (Aub) and Ago3 proteins (reviewed in Blair and Olson, 2014 and Blair and Olson, 2015). Interestingly, in mosquitoes, this pathway seems to have additional functions in generating small RNAs from sources other than transposons (Miesen et al., 2015; Petit et al., 2016) that were initially thought to participate in the mosquito's antiviral defence (Hess et al., 2011; Vodovar et al., 2012; Léger et al., 2013; Schnettler et al., 2013; Miesen et al., 2015, 2016), but recent reports have found that the piwi pathway does not participate in the antiviral response in *Drosophila melanogaster* (Petit et al., 2016). Furthermore, the knockdown of several proteins of the piwi pathway in Aag2 cells infected with DENV 2 did not affect viral replication, suggesting that it has minor participation in the anti-dengue response in insects (Miesen et al., 2016). The population of small RNAs 29 nt in length detected in our analysis likely correspond to piRNAs, and we are performing more studies to corroborate their characteristics and participation during persistent infection.

Deep sequencing and bioinformatics analyses allowed us to identify 37 miRNAs with differential expression in mock-infected, acutely infected, and persistently infected C6/36-HT cells; however, only 15 were regulated in C6-L cells alone compared with the other two conditions. Overall, miR-927, miR-87, miR-190, and miR-970 were over-expressed in C6-L cells compared with the other two conditions. miR-2a-3p, miR-87 and miR-970 were over-expressed to almost the same extent. Interestingly, miR-210 was only found to be up-regulated in C6-L cells compared with acutely infected C6/36-HT cells.

On the other hand, miR-263a-3p, miR-92b, miR-9a-1, miR-9a-5p, miR-286a and miR-286b were down-regulated in C6-L cells compared with the other two conditions tested, and some miRNAs were exclusively down-regulated in C6-L cells compared with acutely infected C6/36 cells: miR-252, miR-10-5p and miR-124. These results suggest that several miRNAs are modulated during both acute and persistent infection, whereas others are specifically modulated during persistent infection.

These results partially correlate with previous findings in acute infections with flaviviruses. For example, the expression of miR-124 was modified in human microglial CHME3 cells during infection with Japanese encephalitis virus (JEV), with it being down-regulated early in infection but over-expressed late in infection (Pareek et al., 2014). The expression of miR-210 is modulated during JEV infection of PK-15 cells (Cai et al., 2015) and over-expressed in *Ae. Aegypti* mosquitoes infected with DENV (Etebari et al., 2015), which correlates with our results. On the other hand, miR-10-5p was up-regulated in *Ae. aegypti* infected with DENV (Etebari et al., 2015) but was down-regulated in C6-L cells in our study. miR-927, miR-87, and miR-2a-3p were down-regulated in *Ae. albopictus* (Liu et al., 2015) and *Ae. aegypti* (Etebari et al., 2015) mosquitoes acutely infected with DENV, but they were up-regulated in C6-L cells in our study. These differences might be related with the fact that C6-L cells are persistently infected with DENV.

4.2. Predicted miRNA targets and their importance in persistent DENV infection

The targets for each miRNA with significant differential expression were predicted using RNAhybrid and Microcosm target software and *Ae. albopictus* transcriptome information (Table 3). Unfortunately, the experimental validation of miRNA targets in mosquitoes is limited, and only a few have been proven (Slonchak et al., 2014; Hussain et al., 2013; Zhang et al., 2013; Yan et al., 2014; Liu et al., 2015). Therefore, the results presented here indicate only putative targets, and further experiments will be required to confirm the role of these miRNAs in the regulation of these genes. These targets included proteins with several activities, including peptidase, scavenger receptor, carbohydrate binding, calcium-dependent phospholipid binding, protein kinase, carboxylic ester hydrolase, ATPase, lysozyme, and translation initiation. Other targets were involved in processes, including ubiquitination, fatty acid biosynthesis, vesicular and protein transport, innate immune and oxidative stress responses, and the JAK-STAT cascade. Finally, others were related to the cytoskeleton or were integral components of the cellular membrane. Here, we focused only on miRNAs that were differentially expressed according to the deep sequencing analysis and then validated by RT-qPCR.

miR-927 was found to be involved in the ubiquitin proteasome pathway (UPP) and probably interacts with two proteins: E3 ubiquitin-protein ligase and a small ubiquitin-related modifier (Table 3). This miRNA was up-regulated in C6-L cells compared with mock- and acutely infected C6/36-HT cells (Figs. 2 and 3), whereas it was previously shown to be down-regulated in *Ae. albopictus* mosquitoes acutely infected with DENV 2 (Liu et al., 2015). The UPP is an important regulator of cellular protein homeostasis that avoids stressing the ER, which could subsequently induce the repression of translation mediated by eIF2α (Choy et al., 2015b; Nag and Finley, 2012). Previous studies have revealed modulation of the expression of genes involved in the ubiquitination machinery in *Ae. aegypti* mosquitoes (Barón et al., 2010; Bonizzoni et al., 2012; Chauhan et al., 2012) as well as in Aag2 cells infected with DENV (Chauhan et al., 2012), including the genes TSG101, NEDD4 (Guo et al., 2010), UBE2A, DDB1, and UBE4B (Choy et al., 2015a). Notably, DENV E and NS5 proteins are able to interact with Ubc9 (Chiu et al., 2007) and UBR4 (Morrison et al., 2013), respectively. Moreover, inhibition of USB14 (Nag and Finley, 2012) or UBE1 (Kanlaya et al., 2010) reduces DENV replication. Silencing UBE2A and DDB1 in *Ae. aegypti* mosquitoes results in a reduction in viral yield but not in the copy number of the viral genome, suggesting that UPP could participate in viral morphogenesis or release (Choy et al., 2015a). This possibility is supported by studies performed in THP-1 cells, which suggest an important role of this pathway in viral particle release (Choy et al., 2015b). Additionally, recent studies performed in A549 cells infected with DENV 2 have revealed that viral genome uncoating is a ubiquitination-dependent but proteasome-independent process (Byk et al., 2016). C6-L cells apparently produce no or very low levels of infectious viral particles even when the viral genome is clearly detected inside the cells (Juárez-Martínez et al., 2013). This phenomenon could be explained, at least in part, by the inhibition of the UPP via over-expression of miR-927. To this end, miR-210, which is also over-expressed in C6-L cells compared with acutely infected cells, may inhibit the expression of a putative furin protease (Table 3). Furin is a *trans*-Golgi resident protease responsible for cleavage of prM into M protein in flaviviruses (Stadler et al., 1997; Keelapang et al., 2014). This proteolysis is an important feature required for the maturation of viral particles that become infectious (Stadler et al., 1997; Perera and Kuhn, 2008). It is possible that the contribution of miR-927 and miR-210 could explain the apparent reduction in viral titre in C6-L cells.

miR-927 may also regulate a putative clathrin-associated protein medium chain. Previous studies have demonstrated that DENV (Mosso et al., 2008; Acosta et al., 2008) and WNV (Chu et al., 2006) use clathrin-mediated endocytosis for entry into C6/36 cells. We speculate that the over-expression of miR-927 in C6-L cells hinders endocytosis to control viral re-infection, but more studies are required to confirm this hypothesis.

Finally, miR-927 also targeted a putative microtubule-associated anchor protein involved in autophagy and membrane

trafficking. Autophagy is a cellular homeostatic process that participates in the degradation and recycling of cytoplasmic components through the formation of double-layered vesicles (Heaton and Randall, 2011; Ghosh et al., 2014; Blázquez et al., 2014), and it is also a component of the innate immune response (Mateo et al., 2013). Although little is known about autophagy during arbovirus infection in insects (Blair and Olson, 2014), it is important during DENV infection and pathogenesis (Blázquez et al., 2014; Lee et al., 2013; Datan et al., 2016), and genes related to its regulation are over-expressed in the midgut of DENV-susceptible strains of *Ae. aegypti* mosquitoes (Chauhan et al., 2012). Inhibition of autophagy results in the generation of non-infectious viral particles in mammalian cells by interrupting viral maturation (Mateo et al., 2013). Additionally, autophagy could be important for lipid metabolism during DENV infection (Heaton and Randall, 2011) or in degradation of components with antiviral activity (Mateo et al., 2013). The association of DENV particles with autophagosomes at early stages after infection has been recently documented, suggesting autophagosome participation during viral replication (Chu et al., 2014). The viral protein NS4A is able to induce autophagy in a PI3K-dependent manner (McLean et al., 2011); ER stress and ataxia telangiectasia mutated (ATM) signalling also induce autophagy (Datan et al., 2016). Finally, several miRNAs involved in autophagy are modulated during infection with JEV in PK-15 cells (Cai et al., 2015). All of these findings show that autophagy is an important process for avoiding cellular death and for promoting viral replication, suggesting its relevance in persistent viral infections. Because miR-927 is up-regulated in C6-L cells, probably resulting in the inhibition of autophagy, this miRNA may be involved in controlling DENV 2 genome replication, reducing the innate immune response and interrupting viral maturation.

In addition to miR-927, three other miRNAs were up-regulated in C6-L cells compared with mock- or acutely infected C6/36-HT cells: miR-87, miR-190 and miR-210 (Figs. 2 and 3).

Putative toll-like receptor 5b is a potential target for miR-87 and miR-190. Additionally, miR-190 could bind to a peptidoglycan-recognition protein transcript (Table 3). Both types of molecules are involved in the innate immune response. Specifically, miR-87 has been implicated in the immune response of *Ae. albopictus* mosquitoes infected with DENV 2 (Liu et al., 2015). The innate immune system of insects is a factor that determines host susceptibility to viral infection (Xi et al., 2008; Ramirez and Dimopoulos, 2010; Carvalho-Leandro et al., 2012); the participation of this system in viral persistence in mosquitoes has been suggested (Goic and Saleh, 2012; Mlera et al., 2014). The innate immune system in insects includes three main pathways, namely Toll, Immune deficiency (IMD), and Janus kinase signal transducer and activator of transcription (JAK-STAT), and the Toll and JAK-STAT pathways are directly involved in the control of DENV infection (Sim and Dimopoulos, 2010; Xi et al., 2008; Souza-Neto et al., 2009). Genes related to the JAK-STAT and Toll pathways are over-expressed in *Ae. aegypti* mosquitoes orally infected with DENV 2 (Xi et al., 2008; Behura et al., 2011), and genes of the Toll pathway are over-expressed in the salivary glands of *Ae. aegypti* mosquitoes infected with DENV (Luplertlop et al., 2011). Additionally, genes related to the immune response are over-expressed in Aag2 cells infected with DENV at an MOI of 1 for 48 h (Sim and Dimopoulos, 2010). Our results suggest that the Toll pathway could be inhibited in persistently infected cells by over-expression of miR-87 and miR-190, allowing for the viral genome to remain in the cell for long periods and participating in the maintenance of persistent infection.

Additionally, the down-regulation of miR-9a-5p, which may be able to interact with putative suppressors of cytokine signalling involved in the JAK-STAT cascade, could contribute to this inhibition of the innate immune response.

miR-87 also may bind to a putative mannose lectin (Table 3). These types of molecules, such as DC-SIGN (Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003) and MR (Miller et al., 2008), have been identified as receptors for DENV infection in mammalian cells. In *Ae. aegypti* mosGCTL proteins, C-type lectin receptors have been described as important determinants of DENV tropism, especially mosGCTL-3 (Liu et al., 2014), and C-type Lectin AAEL011453 is over-expressed in the midgut of strains of *Ae. aegypti* mosquitoes that are refractory to DENV (Chauhan et al., 2012). Although the function of these types of molecules as DENV receptors in C6/36-HT cells should be determined, we speculate that the up-regulation of miR-87 might restrict its expression in order to control viral infection. More intriguing is the putative interaction of miR-87 and miR-210 with proteins with scavenger activity (Table 3). DENV is able to infect Huh-7 and U937 cells through scavenger receptor class B type I (SR-BI) by its interaction with apolipoprotein A-I, which is the main component of high-density lipoprotein (HDL) in human serum (Li et al., 2013), but the significance of this interaction in mosquito cells is unknown.

miR-190 and miR-210 could also interact with mRNAs encoding proteins with endopeptidase activity (Table 3). Serine (Ser) proteases are enzymes involved in processes of digestion and immunity (Bonizzoni et al., 2012). Modulation of the activity of these enzymes has been described during DENV infection (Bonizzoni et al., 2012). The clip domain Ser proteases (clip-SPs) are characterised by the presence of a 30–60 amino acid clip domain, and they are involved in early embryogenesis and the innate immune response in insects, including Toll pathway activation and melanisation. In *Drosophila melanogaster,* clip-SPs are involved in the proteolytic activation of Späzel and phenoloxidase, which activate the Toll pathway and the melanisation process, respectively (reviewed in Jang et al., 2008). Aag2 cells treated with heat-inactivated DENV 2 presented an over-expressed clip-SP and serine protease inhibitor (serpin) and several down-regulated serine proteases (Sim and Dimopoulos, 2010). At 10 days after DENV 2 infection in *Ae. aegypti* mosquitoes, the activation of serpins, such as SRPN4A, SRPN4D, and SRPN21, and down-regulation of SRPN9 and SRPND10 have been reported (Xi et al., 2008), suggesting their participation in DENV infection, although their specific roles are still unknown. Additionally, miR-9a-5p may be able to interact with a putative serine-type endopeptidase inhibitor. Overall, the over-expression of miR-190 and miR-210 and down-regulation of miR-9a-5p in C6-L cells (Figs. 2 and 3) may regulate the expression and activity of these Ser proteases and serpins in order to control the innate immune response during persistent DENV infection in C6-L cells. However, further experiments are required to clarify such interactions.

Additionally, miR-210 could interact with the mRNA encoding a protein with lysozyme activity (Table 3). Lysozymes are antimicrobial peptides that affect microbiota in the midgut of mosquitoes; these peptides may participate in immune activation (Ramirez et al., 2012). For example, expression of LYSC7B (AAEL015404) was differentially regulated in *Ae. aegypti* mosquitoes orally inoculated with DENV 2 (Xi et al., 2008); LYSC11 (AAEL003723) was more abundant in non-infected *Ae. aegypti* mosquitoes than in those orally infected with DENV 2 (Bonizzoni et al., 2012); and Lysozyme C expression was significantly elevated in the midgut under similar experimental conditions. This result correlates with an increase in viral yield after Lysozyme C silencing (Ramirez et al., 2012). Lysozyme P is over-expressed in the salivary glands of *Ae. aegypti* mosquitoes infected with DENV (Luplertlop et al., 2011), and Lysozyme G is down-regulated in the midgut and over-expressed in body fat at 24 h post-infection and then down-regulated 3 days later (Ramirez and Dimopoulos, 2010). The up-regulation of miR-210 probably inhibits the expression of Lysozyme C-4 in C6-L cells, a peptide that is activated by the IMD pathway (Xi et al., 2008), thus

establishing the conditions for persistent viral infection through its interaction with the immune system.

miR-124 is down-regulated in C6-L cells (Fig. 3B), and its putative targets are a low-density lipoprotein receptor (LDLr) and phospholipid-transporting ATPase (Table 3), suggesting its role in lipid metabolism. Lipidomic studies have revealed an increase in sphingolipid and phospholipid synthesis in DENV-infected C6/36 cells, with important effects on membrane remodelling. Interestingly, ceramide, a sphingolipid related to autophagy, was increased in these cells (Perera et al., 2012). Additionally, increased expression of enzymes involved in fatty acid biosynthesis has been described in DENV 2-infected Aag2 cells (Sim and Dimopoulos, 2010). Finally, DENV4 infection of Huh-7 cells induces the translocation of the LDLr to the cell surface to increase the uptake of LDL particles (Soto-Acosta et al., 2013). Interestingly, miR-10-5p, which is also involved in the fatty acid biosynthesis pathway, was down-regulated in C6-L cells (Fig. 2). This modification in lipid metabolism might be associated with the formation of double-membraned vesicles that are important for the assembly of viral replicative complexes (Junjhon et al., 2014), indicating the possible participation of this miRNA in regulating viral replication inside the cell. Moreover, miR-124 also could interact with translation initiation factor eIF3a, which participates in the binding of initiation tRNA and mRNA to the 40S ribosomal subunit in eukaryotes (Bommer et al., 1991) as well as in prokaryotes (Betney et al., 2010) but also enhances the removal of tRNA from the ribosomal P-site (Antoun et al., 2006) to promote ribosomal recycling (reviewed in Seshadri and Varshney 2006). Adequate control of the expression of eIF3a is required to avoid premature termination of the translation process (Betney et al., 2010). The DENV genome is translated in a cap-dependent but also cap-independent manner where the factor eIF4E is dispensable (Edgil et al., 2006). The down-regulation of miR-124 could permit the functionality of the translation process, which is required for all cellular protein biosynthesis, to maintain cellular viability during infection.

In addition to its interaction with a putative serine-type endopeptidase inhibitor and suppressors of cytokine signalling in the JAK-STAT cascade, miR-9a-5p could interact with a putative vesicle coat complex COP II subunit sfb3 involved in endoplasmic reticulum (ER)-Golgi apparatus vesicle-mediated transport. The COP II complex participates in the anterograde transport of vesicles from the ER to the Golgi apparatus (Brandizzi and Barlowe, 2013). Previous studies have reported the over-expression of genes involved in protein transport into the ER in Aag2 cells infected with DENV 2 for 48 h, suggesting an increase in protein transport during infection (Sim and Dimopoulos, 2010). The presence of vesicles attached to the ER membrane, where viral replication and assembly occur, has been observed during the replicative cycle of DENV (reviewed in Mukhopadhyay et al., 2005 and Nagy and Pogany, 2012), and the transport of viral particles through the Golgi apparatus plays an important role in virion maturation and release (Rodenhuis-Zybert et al., 2010). Because miR-9a-5p is down-regulated in persistently infected cells, such expression could correlate with adequate intracellular trafficking of proteins, which is required for viral replication and maintenance of the viral genome for long periods.

Finally, a previous study has shown that miR-252 was over-expressed in C6/36 cells infected with DENV 2 for 24 and 72 h. This miRNA has putative targets in the E protein of DENV 2 and 3 but apparently none in DENV 1 or 4. Inhibiting miR-252 results in an increase in both, the number of viral genomes and virus titres, suggesting the direct participation of this miRNA in the regulation of DENV replication (Yan et al., 2014). Interestingly, this miRNA was slightly down-regulated in C6-L cells compared with acutely infected cells (Fig. 2), suggesting a mechanism induced by the virus to remain in the cells for long periods of time. However, this assumption will require further confirmation.

5. Conclusions

The results presented in this work suggest that the expression of several miRNAs is regulated during persistent infection with DENV in C6/36-HT mosquito cell line, indicating the participation of cellular factors in establishing and maintaining infection.

The expression of several miRNAs was modulated in C6-L cells in comparison with mock-infected or acutely infected C6/36-HT cells. Almost all were shared when both conditions were compared with C6-L cells, and only four were modulated in C6-L cells compared with one condition: miR-210 was overexpressed and miR-252 and miR-124 were down-regulated in C6-L compared with acutely infected C6/36-HT cells. Only miR-286b was down-regulated in C6-L compared with mock-infected cells.

Differential expression of miR-927, miR-9a-5p, miR-190, miR-87, miR-124 and miR-210 was validated by RT-qPCR. In the case of miR-190 and miR-87, significant differential expression was found only compared with mock-infected cells.

The putative targets of these miRNAs were components of ubiquitin conjugation and the immune innate response; proteins with endopeptidase, kinase, or Lectin C-type activity; or proteins that participate in processes such as autophagy, protein transport, and fatty acid biosynthesis. All of these processes are important during DENV infection.

This work offers, for the first time, an overview of several miRNAs that are differentially expressed in a persistently infected mosquito cell line with DENV, and the pathways and cellular factors that could be involved in the maintenance of this persistent infection. However, studies that are more detailed will be required to confirm their participation in the model of a cell line as well as in the context of the whole mosquito. In our laboratory we are currently performing the functional experiments to demonstrate the relevance of those miRNA in the process of the DENV persistent infection.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2017.03.005.

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