**Wolbachia-Induced aae-miR-12 miRNA Negatively Regulates the Expression of MCT1 and MCM6 Genes in Wolbachia-Infected Mosquito Cell Line**

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**Abstract**

**Background:** Best recognized for its role in manipulating host reproduction, the parasitic gram-negative *Wolbachia pipientis* is known to colonize a wide range of invertebrates. The endosymbiotic bacterium has recently been shown to cause a life-shortening effect as well as inhibiting replication of arboviruses in *Aedes aegypti*; although the molecular mechanisms behind these effects are largely unknown. MicroRNAs (miRNAs) have been determined to have a wide range of roles in regulating gene expression in eukaryotes. A recent study showed that several *A. aegypti* mosquito miRNAs are differentially expressed when infected with *Wolbachia*.

**Methodology/Principal Findings:** Based on the prior knowledge that one of these miRNAs, aae-miR-12, is differentially expressed in mosquitoes infected with *Wolbachia*, we aimed to determine any significance of this mediation. We also set out to characterize the target genes of this miRNA in the *A. aegypti* genome. Bioinformatic approaches predicted a list of potential target genes and subsequent functional analyses confirmed that two of these, DNA replication licensing (MCM6) and monocarboxylate transporter (MCT1), are under the regulative control of aae-miR-12. We also demonstrated that aae-miR-12 is critical in the persistence of *Wolbachia* in the host cell.

**Conclusions/Significance:** Our study has identified two target genes of aae-miR-12, a differentially expressed mosquito miRNA in *Wolbachia*-infected cells, and determined that the miRNA affects *Wolbachia* density in the host cells.

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**Introduction**

*Wolbachia* is an endosymbiotic gram-negative bacterium which is estimated to infect as many as two thirds of all insect species [1]. This often parasitic endosymbiont is most commonly associated with manipulating host reproductive strategies through means such as feminization and cytoplasmic incompatibility [2]; although some strains of the bacterium have been known to have mutualistic or commensal interactions with their hosts [3].

In 2008, McMeniman et al. introduced the αMelPop-CLA strain of *Wolbachia* into *Aedes aegypti*, which is the main vector of dengue viruses. This had the effect of reducing the adult female’s lifespan by as much as 50% [4]. In addition to the life-shortening effect, *Wolbachia* infection of *A. aegypti* was shown to inhibit replication of several pathogens, including dengue viruses [5]. Despite the significance of these findings, relatively little is known about the molecular mechanisms which mediate the changes made to the mosquito’s biology upon infection with *Wolbachia*.

Since their discovery in 1993 [6], microRNAs (miRNAs), as ~22 nucleotide non-coding RNAs, have been credited with an ever-expanding range of roles in regulating expression of genes. Seemingly specific to eukaryotes, their initial discovery in round worms illustrated the role of miRNAs in post-transcriptional control over genes responsible for developmental timing [7]. Since then, miRNAs have been implicated in many more biological processes [8]. There are multiple different modes of miRNA actions currently described which rely on the interaction of miRNAs with their target miRNAs [9]. Binding of a miRNA to its target mRNA may lead to repression of translation, degradation of target transcripts or even up-regulation of transcript levels [10,11].

MiRNAs can also play a part in interactions between organisms. One such scenario was described in the case of *Helicobacter pylori*. It was demonstrated by Fehri et al. (2010) that infection with *H. pylori* induces the expression of miR-135 and down-regulates miR-218 [12]. This overall manipulation of host miRNAs results in an alteration to metabolic pathways within the cell. Ultimately, this leads to an increase in gastric tumours [12]. In another example, it was shown that *A. aegypti* miRNA expression profile is altered when infected with *Wolbachia* [11]. The study only identified the target of aae-miR-2940, which was one of the miRNAs that showed up-regulation upon *Wolbachia* infection [11]. Despite many well-demonstrated examples such as these, there still exist large gaps in the current state of knowledge regarding the exact role of miRNAs.
in host-pathogen interactions, especially in invertebrates [13]. Notably, in the case of A. aegypti whose miRNA profile is altered in response to infection with the parasitic aMelPop-CLA strain of Wolbachia [11], very few of the gene targets of these differentially expressed miRNAs have been identified.

Using bioinformatic techniques to predict possible targets and qRT-PCR to quantify the mRNA transcript levels of those targets across various treatments, we aimed to characterize the gene targets(s) of aae-miR-12 which was found to be differentially expressed in A. aegypti mosquitoes infected with Wolbachia when the previous microarray data [11] were further analyzed. Results from this study demonstrated that the A. aegypti genes, DNA replication licensing factor (MCM6) and monocarboxylate transporter (MCT1), were significantly down-regulated in response to Wolbachia infection. Further experimentation in insect cell lines concluded that this was a direct result of the up-regulation and subsequent interaction of aae-miR-12 with the targets. In addition, results suggested that aae-miR-12 is essential for Wolbachia replication/maintenance in the host cells.

Results

Bioinformatics Predicts MCM6, MCT1 and Exonuclease as Targets for aae-miR-12

NCBI BLAST searches were utilized to generate a list of homologies between the aae-miR-12 and genes within the A. aegypti genome. This approach produced a shortlist of 32 genes as potential targets of the miRNA. Subsequently, these genes were individually evaluated using RNASHift miRNA target prediction software, which produced a refined list of three most likely targets as shown in Table 1. These genes had predicted target sites in their open reading frames with substantial complementary sequences to the seed region or the rest of aae-miR-12 as well as a free energy of interaction which were calculated to be greatly negative; −27.6 kcal/mol for MCM6, −30 kcal/mol for Exonuclease and −27.2 kcal/mol for MCT1 (Figure 1).

Wolbachia Induces Suppression of MCM6 and MCT1 Genes in A. aegypti Cells

Given the knowledge that aMelPop-CLA infected mosquitoes showed significant induction of aae-miR-12 when compared to uninfected mosquitoes [11], we tested the hypothesis that this miRNA has a regulatory effect on A. aegypti target genes predicted using bioinformatics analysis, which in turn allows the bacterium to colonize its host more effectively. Using primers predicted using bioinformatic techniques to predict possible targets and qRT-PCR to quantify the mRNA transcript levels of those targets across various treatments, we aimed to characterize the gene targets(s) of aae-miR-12 which was found to be differentially expressed in A. aegypti mosquitoes infected with Wolbachia when the previous microarray data [11] were further analyzed. Results from this study demonstrated that the A. aegypti genes, DNA replication licensing factor (MCM6) and monocarboxylate transporter (MCT1), were significantly down-regulated in response to Wolbachia infection. Further experimentation in insect cell lines concluded that this was a direct result of the up-regulation and subsequent interaction of aae-miR-12 with the targets. In addition, results suggested that aae-miR-12 is essential for Wolbachia replication/maintenance in the host cells.

Table 1. Predicted targets of aae-miR-12.

| Accession Number | Gene Function            |
|------------------|--------------------------|
| AaeL_AAEL007801  | Exonuclease              |
| AaeL_AAEL005246  | DNA replication licensing factor MCM6 |
| AaeL_AAEL002412  | Monocarboxylate transporter MCT1 |

doi:10.1371/journal.pone.0050049.t001

Aae-miR-12 Transfection Mimics the Effect of Wolbachia in Regards to MCM6 and MCT1 Regulation

To confirm that down-regulation of MCM6 and MCT1 in Wolbachia-infected Aag2 cells was a result of aae-miR-12 interaction with the target genes, additional experiments were conducted transfecting Aag2 uninfected cells with the synthetic aae-miR-12 mimic. As controls, cells were mock-transfected or transfected with a control mimic, which consisted of random sequences. Aag2 cells that were transfected with the synthetic aae-miR-12 mimic showed significantly reduced transcript levels for both the MCM6 and MCT1 genes 72 h post-transfection (Figure 2). Aag2-aMelPop-CLA cells transfected with the control mimic showed little observable difference to their mock-transfected counterparts (Figure 2A, C; p>0.05). To further confirm the specific interaction of aae-miR-12 with the targets, two mutant mimics of aae-miR-12, with point mutations in the regions where substantial sequence complementarity with the target exists (see Material & Methods for details), were used in similar but independent experiments. However, no significant reduction in the transcript levels of MCM6 or MCT1 was observed (Figure 3B, D; p>0.05).

Subsequently, we proceeded to test the hypothesis that if indeed aae-miR-12 is responsible for the down-regulation of MCM6 and MCT1 then transfection of a synthetic aae-miR-12 inhibitor would rescue the miRNA transcript levels of these genes in Wolbachia-infected cells. Results indicated exactly this in the case of aag2-aMelPop-CLA cells which had previously been shown to have significantly suppressed transcript levels of both MCM6 and MCT1 (Figure 4A, B; p = 0.000249, p = 0.000324). In these cells, transfection with the synthetic aae-miR-12 inhibitor decisively up-regulated the MCM6 and MCT1 transcripts. The control inhibitor had no effect on the transcript levels of either of the genes (Figure 4A, B; p>0.05).

In order to determine whether the results achieved in vitro were consistent with similar processes in vivo, the transcript levels of MCM6 and MCT1 in total RNA extracted from whole female mosquitoes (4 days after emergence) were determined. The results from these experiments revealed that there was significantly lower transcript levels of MCM6 (Figure 5A; p = 0.000209), and reduced levels of MCT1 (Figure 5B) in Wol mosquitoes compared to uninfected—Wol mosquitoes; although the difference in the case of MCT1 was not statistically significant (p = 0.109) as in the cell line.

Aae-miR-12 Positively Interacts with MCM6 and MCT1 Target Sites in Sf9 Cells

To test the interaction of aae-miR-12 with ORF target sites of both the MCT1 and MCM6 genes, both target sites were cloned downstream of a GFP reporter gene in the commercially available pLZ/V5 vector. These were subsequently co-transfected into Sf9 cells (derived from Spodoptera frugiperda) together with a control mimic or aae-miR-12 mimic. The expression of GFP in Sf9 cells using the vector is optimal and also the cell line provides an independent system to test the miRNA-target interaction.
Using primers specific to the GFP reporter sequence, qRT-PCR analyses were conducted to assess the effect of miRNA-mRNA interaction on the GFP transcript levels. It was determined that whilst very little difference could be observed in the levels of GFP reporter transcripts between the mock and the control mimic treatments (p>0.05), there were significantly higher levels of GFP transcripts in those cells transfected with the aae-miR-12 mimic (Figure 6; p=0.003, p=0.006). This was the case for both the pIZ-GFP-MCT1 (Figure 6A) and pIZ-GFP-MCM6 (Figure 6B) constructs.

Figure 1. The A. aegypti MCM6, Exoculease and MCT1 were predicted to be the best targets of aae-miR-12 with significant sequence complementarities.
doi:10.1371/journal.pone.0050049.g001

Figure 2. qRT-PCR analysis of predicated target genes of aae-miR-12 in aag2-wMelPop-CLA and uninfected Aag2 cells. The error bars indicate standard deviations of averages from two biological and three technical replicates. ***, p<0.001; ns, p>0.05.
doi:10.1371/journal.pone.0050049.g002
Inhibition of aae-miR-12 Reduces the Wolbachia Density in Mosquito Cell Line

Given that Wolbachia infection regulates the expression of aae-miR-12 in mosquitoes, we hypothesised that this miRNA has a crucial role in mediating the presence of cellular proteins, which serve to further the bacterium’s ability to persist in the cells. Equal numbers of aag2-w MelPop-CLA cells were mock-transfected, transfected with the synthetic aae-miR-12 inhibitor or the control inhibitor. Using the primers specific to wsp gene (normally used to quantify Wolbachia density), a qPCR was undertaken on genomic DNA extracted from each of the treatments 72 h after the transfection. The results indicated that as opposed to the mock and the control inhibitor treatments, aae-miR-12 inhibitor substantially reduced the density of Wolbachia in the host cells (Figure 7; p 0.0049).

Discussion

Although many efforts have been dedicated to understanding the biology of Wolbachia, there still exists a plethora of unexplored mechanisms underlying its interactions with other organisms, through which it appears to be manipulating its host’s environment in an attempt to insure the bacterium’s survival. The findings of Hussain et al. (2011) [11] have shed light on what appears to be one of the molecular mechanisms by which Wolbachia mediates changes in the host A. aegypti. The functional analyses carried out in the report demonstrated that mosquito cellular miRNAs are differentially expressed as a result of Wolbachia infection, a finding consistent with current literature, which describes similar phenomena in other host-pathogen interactions across Eukarya [2,14,15]. One such example in humans is infection with H. pylori, which induces miR-155 in T cells [12]. Hussain et al. (2011) went on to further illustrate the specific role of one of these differentially expressed miRNAs, aae-miR-2940, in regulating a metalloprotease cellular protein, which is imperative in facilitating Wolbachia’s colonization of its mosquito host [11].

In this study, the regulatory role of another differentially expressed mosquito miRNA, aae-miR-12, which is up-regulated by Wolbachia, is described. Based on bioinformatics predictions, we investigated the miRNA-mRNA interactions of aae-miR-12 and three characterized mosquito target genes. Quantitative-Real Time PCR analyses of levels of mRNA transcripts revealed that both the MCT1 and MCM6 genes were suppressed by this miRNA in vitro and in vivo. However, there was no effect on Exonuclease
cells with the miRNA inhibitor of aae-miR-12, it was discovered regulation of these genes. Upon transfection of Wolbachia-aae-miR-12 in regulation of artificially inducing aae-miR-12 could mimic logical prediction generated by the experiment that if indeed the case. This finding was further scrutinized by testing the PLOS ONE | www.plosone.org 5 November 2012 | Volume 7 | Issue 11 | e50049 group with the same letter at standard deviations of averages from two biological and three technical doi:10.1371/journal.pone.0050049.g004 and MCM6 transcript levels; therefore, further studies concentrated on MCT1 and MCM6 target genes. In addition, further functional analyses demonstrated the crucial role aae-miR-12 plays in Wolbachia’s fitness in the mosquito cell line tested.

Confirmation of the interaction of aae-miR-12 with the predicted targets was first attempted through the use of a synthetic aae-miR-12 mature miRNA mimic in cells not infected with Wolbachia to see if aae-miR-12 alone could mimic Wolbachia’s effect on MCT1 and MCM6 genes. The results indicated that this was indeed the case. This finding was further scrutinized by testing the logical prediction generated by the experiment that if indeed artificially inducing aae-miR-12 could mimic Wolbachia’s down-regulation of MCT1 and MCM6 genes, then conversely inhibiting aae-miR-12 in Wolbachia-infected cells should reverse the down-regulation of these genes. Upon transfection of Wolbachia-infected cells with the miRNA inhibitor of aae-miR-12, it was discovered that this treatment could in fact prevent Wolbachia from down-regulating MCT1 and MCM6, providing further evidence in support of the hypothesis that aae-miR-12 interacts with the respective predicted target sites in both of the target genes. Surprisingly, when GFP was used as a reporter gene in the pIZ vector, downstream of which target sites for both MCT1 and MCM6 genes, respectively, were cloned and then transfected into Sf9 cells, the synthetic aae-miR-12 mimic had the effect of significantly increasing GFP transcript levels rather than decreasing them. This finding is directly opposite to the trends observed in A. aegypti cells. Upon further inspection of the literature however, it appears that this phenomenon is not undescribed. For example, Callis et al. (2009) also showed that miRNA can have opposing effects on transcript levels depending on the miRNA-reporter binding context [16]. In this instance, the context of interaction which is downstream of GFP differs from the interaction in target gene mRNAs in that the target sites for MCT1 and MCM6 genes are in the coding region. In this experiment however, in the GFP construct the target sites become localized to what effectively becomes the 3’UTR of the GFP transcript, thus the nature of the interaction is altered and produced a different outcome. These findings may lend themselves to the hypothesis that aae-miR-12 is interacting with target sites in both MCT1 and MCM6, but the fact that the nature of the interaction differs depending on the cellular circumstances highlights the detail that the precise method of transcript regulation is still elusive. As miRNAs can interact with their targets in many ways, this represents an interesting avenue for further research into these specific interactions.

Considering that cell lines represent in vitro experimental systems and may not necessarily be representative of in vivo processes affecting overall mosquito’s physiology, we undertook quantitative experimentation on RNA extracted from whole mosquitoes to determine the overall expression of MCM6 and MCT1 in vivo in+Wol and –Wol mosquitoes. Results of these experiments showed the same pattern of expression for our predicted target genes across the treatments as we observed in cell lines. In mosquitoes infected with Wolbachia, we observed significantly lower levels of MCM6 transcripts than in uninfected mosquitoes and whilst we saw a similar reduction in the case of MCT1, the reduction was not statistically significant. This is likely due to the fact that MCT1 is not expressed in all mosquito tissues, which would imply that the transcript reduction effect would be diluted in a total RNA extraction. This is not the case in a clonal cell culture in which all cells may express the gene.

We derived the hypothesis that if indeed Wolbachia had a role to play in regulating cellular proteins through induction of aae-miR-12, then inhibiting this process would somehow have a detrimental effect on the ability of Wolbachia to persist in the host cell. In order to test this hypothesis, we transfected Aag2 cells infected with Wolbachia with aae-miR-12 inhibitors and measured the effect this had on Wolbachia density using the wsp gene as an indicator. qRT-PCR results showed that whilst a control inhibitor with scrambled sequence had little effect on the number of detectable copies on the wsp gene in Wolbachia-infected cells, transfection of the aae-miR-12 inhibitor significantly reduced Wolbachia density. This suggests that aae-miR-12 plays a critical role in modifying the cellular environment of the endosymbiont’s host in order to increase that environment’s habitability for Wolbachia by changing the levels of MCM6 and MCT1 proteins within the cell.

The Drosophila melanogaster homologue of MCM6 has been demonstrated to play a critical role in chorion gene amplification as well as genomic replication [17]. This function in coding a DNA replication licensing factor has been well documented and

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**Figure 4. Validation of aae-miR-12 interaction with MCT1 and MCM6 target genes using miRNA inhibitors.** qRT-PCR analysis of RNA extracted from aag2-wMelPop-CLA cells mock-transfected, transfected with aae-miR-12 inhibitor and control inhibitor and analysed with specific primers to (A) MCM6 and (B) MCT1. The error bars indicate standard deviations of averages from two biological and three technical replicates. There are no statistical significant differences within the group with the same letter at $p>0.05$. doi:10.1371/journal.pone.0050049.g004
attributed to MCM6 across a broad range of eukaryotes [18]. This is a strong indication that this gene is well conserved across eukaryotes. Also in Drosophila, MCT1 encodes a monocarboxylate transporter which serves to provide metabolic fuel such as lactate and pyruvate to cells [19]. Exactly what function or specific benefit may be conferred to Wolbachia by suppressing MCM6 and MCT1 genes in host cells is as of yet unclear and requires further investigations.

In conclusion, we have demonstrated the importance of the cellular miRNA aae-miR-12 in Wolbachia colonization of A. aegypti Aag2 cells. Our results demonstrated that inhibition of this miRNA drastically reduces Wolbachia’s persistence in host cells. Furthermore, we have identified potential targets of this miRNA through bioinformatics techniques. These targets, MCM6 and MCT1 genes, were functionally validated and demonstrated to be under the regulative control of aae-miR-12 both in vitro and in vivo. The findings demonstrated here merit further study into...
the precise role the proteins encoded by these target genes play in mediating host-pathogen interactions.

Materials and Methods

Maintaining Mosquitoes and Cell Lines

Previously, McMeniman et al. (2008) [4] had generated mosquitoes and cell lines infected with aMelPop-CLA strain of Wolbachia and also those cured with tetracycline, which were utilized in the experiments. For those cells infected with aMelPop-CLA, the procedure of infection is described by Frentiu et al. (2010) [20]. PCR assays were conducted to ensure stable infection continued throughout experimentation. Aag2 cells derived from A. aegypti were cultured in Schneider’s medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS).

miRNA Target Predictions

The A. aegypti genome was screened for potential targets bearing homology to aae-miR-12 using NCBI BLAST. A list of 32 potential targets was generated this way. These candidates were then analysed with the RNAHybrid and RNA22 prediction software to confirm their potential interaction with aae-miR-12 as well as all possible binding sites for the miRNA. From this information, a refined target list of 3 different genes was compiled (Table 1).

Inhibition and Mimic of aae-miR-12 in Mosquito Cell Line

The aae-miR-12 inhibitor (reverse complement RNA oligo) and mimic (UGAGUAAUACAUCAUCAGGUUGU) were synthesized by Genepharma along with control “scramble” inhibitor (UCUACUGUUUCUAGGAAUGUGA) and mimic (UU-CUCGGAAGGUAGUGAGT). In addition, two mutant mimics with two nucleotide mutations in the complementary regions with the targets were synthesized and used in transfections (mutant mimic-1 UGAGUAAUACAUCAUCAGGUUGU and mutant mimic 2 UGAGUAAUACAUCAUCAGGUUGU; mutated residues are underlined). Transfection of inhibitors and mimics was undertaken using 2 µg of RNA. Cellfectin was used as the transfection reagent according to the manufacturer’s instructions (Invitrogen). Total RNA/DNA extraction from these cells was performed 72 hours post-transfection.

qPCR Analyses

Total RNA was extracted from cells using TRI Reagent according to the manufacturer’s instructions (Molecular Research Centre). The concentration of RNA was measured using nanodrop. Dilutions of 100 ng/µl RNA were then produced for further experimentation. Gene-specific reverse primers were used in conjunction with reverse transcriptase Superscript II (Invitrogen) to generate cDNA to be subsequently amplified in qPCR reactions. The temperature conditions for the 1st strand synthesis began with annealing the primers to the RNA template at 65°C for 5 min followed by 1 h at 30°C. Both forward and reverse primers specific to the target gene being tested were used for qPCR reactions. Each qPCR reaction was performed in triplicates in two biological replicates. qPCR primers used for MCM6 were MCM6-For 5’-GGAGGCTTTGCAAGATGCGGGGA-3’ and MCM6-Rev 5’-CGTGGCAACCGAA-GACGGCCA-3’, and for MCT1 were MCT1-For 5’-CACCCCGTGGCTTACCCCGG-3’ and MCT1-Rev 5’-AGCCCCATCCACCATTGGGT-3’. The qPCR cycling conditions were 94°C for 3 min, 94°C for 15 sec, 60°C for 40 sec and 72°C for 45 sec, repeated for 36 cycles. For qPCR reactions using genomic DNA as the template a 2 min melt step was added and the melt curve was analysed to ensure amplification was a uniform product. Melting curves were analyzed after each run to check the specificity of amplification. The relative ratio of MCM6 and MCT1 mRNAs to cellular RNA (RPS17 used for normalizing data) was determined using the specific primers given above and RPS17 specific primers (rps17-For 5’-CACTTCGGAGTGTCCTGGTGATGAT-3’ and rps17-Rev 5’-GGACACTTGCGCCCCGACGTAGT-3’).

DNA extraction was performed using a homogenization mixture as per Invitrogen instructions, and primers specific to the Wolbachia housekeeping gene Wsp were wsp-For 5’-GTCTAAGTTATSTGATAGCCAGAC-3’ and wsp-Rev 5’-GTTGCCACCAA-CAGGCTTATAAA-3’. All qRT-PCR data was subjected to statistical analyses using ANOVA and t-tests followed by Tukey’s test where relevant.

Cloning Targets Under GFP in pIZ

Fragments approximately 250 bp long from both MCM6 and MCT1 containing the target sequences of aae-miR-12 were amplified from total A. aegypti RNA using primers that were designed to amplify these fragments with specific restriction sites XbaI and SacII (New England Biosciences) for cloning into the pIZ vector. These primers were qMCM6-For 5’-GTCTAGAAGTTGATTGTCTTGCGGCTGAAA-3’, qMCM6-Rev 5’-GCGCGGGGCCCCAGGAGGCGCATCTTGTGAT-3’ for MCM6 and qMCT1 For 5’-GTTCTAGATGCTGTGAGACGATCAGATTGGAAGATTGCGGATTGTA3’ for MCT1. The fragments containing the restriction sites were then purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen) and ligated into the plZ vector (Invitrogen) containing GFP. Vectors which could be digested with EcoRI and SacII to liberate fragments of the predicted size were sequenced to confirm the identity of the cloned fragments. Transfection of these plasmids into insect cells.
was performed using 2 μg of the plasmid per transfection. As a control, cells were transfected with pIZ-vector containing only GFP. Cellfectin was used as the transfection reagent according to the manufacturer’s instructions (Invitrogen). Primers specific to the GFP sequence (GFP-For 5’-CCCAAGCTTCGCACTGGTGAGC-3’ and GFP-Rev 5’-GGGGGTACCCTGACTACGAGTGACG-3’) were used for the subsequent qRT-PCR 72h after transfection. Data were analysed as above using actin as the normalizing gene. The primers used for actin were actin-For 5’-ATGGAGAAGATCTTGCAC-3’ and actin-Rev 5’-GGAGCCTCGTGAGCAGC-3’.

Author Contributions
Conceived and designed the experiments: SA MH. Performed the experiments: SO-A. Analyzed the data: SO-A MH SA. Contributed reagents/materials/analysis tools: SA SLO. Wrote the paper: SO-A SA.

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