LETTER

Reply to Skalsky et al.: A microRNA-like small RNA from Dengue virus

Skalsky et al. (1) indicate that viral small RNA (vsRNA)-5 (2) was not found in previous deep sequencings, is present in very low abundance, and likely is an RNA interference (RNAi) product. With advancements in next-generation sequencing, progressively deeper sequencing results are obtained. In fact, vsRNA-5 reads were present in the data from the recently published research in which *Aedes aegypti* mosquitoes were infected with Dengue virus 2 (DENV2) (3), although the infection rate in mosquitoes was only 50%. Evidence shows that there is bias in profiling small RNAs using deep sequencing, and therefore numbers may not show the accurate composition of the profile (4). Differences may also exist in the abundance of microRNAs (miRNAs) between whole mosquitoes with heterogeneous tissues and cell line. The abundance of vsRNA-5 was high enough in DENV-infected Aag2 cells to be detected by Northern blot and cloned through a miRNA-specific RT-PCR technique.

vsRNA-5 does not appear to be an RNAi degradation product because (*i*) silencing of Dicer-2 (involved in RNAi) did not affect vsRNA-5 levels, whereas Dicer-1 did; (*ii*) vsRNA-5 was detectable, using Northern blotting, in C6/36 cells shown by independent studies, including a couple of authors of the Letter, to be RNAi deficient (5, 6); (*iii*) the probe to the other part of the stem-loop did not detect any small RNA; and (*iv*) vsRNA-5 could be produced from the precursor stem-loop either cloned in the pIZ vector or from single-stranded RNA (ssRNA) after transfection into Aag2 cells.

In terms of function, the effect of vsRNA-5 on DENV was specific, as inhibitors to the other five vsRNAs had no effect or, opposite to vsRNA-5, slightly reduced DENV levels. Inhibitors of vsRNAs were transfected into Aag2 cells before virus exposure meaning that the vsRNA-5 inhibitor was present all throughout the infection, perhaps leading to a big impact on DENV levels; nevertheless, a specific effect was observed with vsRNA-5 that was not seen with the other vsRNA inhibitors. Of relevance here, about the comment on the poor effect of simple reverse complementary inhibitors on targets, inhibitors/antagomirs with reverse complementary sequences and 2′*O*-methylation modification, used in this study, are routinely used in miRNA studies and have been proven effective. Further to the specificity of vsRNA-5: target interaction, in contrast to the vsRNA-5 mimic, the vsRNA-6 mimic used as a control had no effect on DENV levels or ssRNA containing NS1 target sequences. vsRNA-5: target sequence interaction was also validated by GFP reporter constructs, and a specific suppressive effect of the vsRNA-5 mimic on NS1 (binding to its ORF) was observed at the RNA and protein levels when overexpressed in Aag2 cells.

Finally, miRNAs are known as fine tuners found in high or low abundance, which may vary over time. Several virus-encoded miRNAs are known that target viral genes to regulate virus replication. In a mosquito vector, a low but transmissible level of replication is desirable to avoid compromising the host's fitness. Therefore, low-level expression of a regulatory vsRNA to keep virus levels in check is considered beneficial to the virus; hence, mutations in vsRNA-5 or its target may be detrimental to the vector.

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1 Skalsky RL, Olson KE, Blair CD, Garcia-Blanco MA, Cullen BR (2014) A "microRNA-like" small RNA expressed by Dengue virus? *Proc Natl Acad Sci USA* 111:E2359.

2 Hussain M, Asgari S (2014) MicroRNA-like viral small RNA from Dengue virus 2 autoregulates its replication in mosquito cells. *Proc Natl Acad Sci USA* 111(7):2746–2751.

3 Campbell CL, Harrison T, Hess AM, Ebel GD (2014) MicroRNA levels are modulated in *Aedes aegypti* after exposure to Dengue-2. *Insect Mol Biol* 23(1):132–139.

4 Sun G, et al. (2011) A bias-reducing strategy in profiling small RNAs using Solexa. *RNA* 17(12):2256–2262.

5 Brackney DE, et al. (2010) C6/36 *Aedes albopictus* cells have a dysfunctional antiviral RNA interference response. *PLoS Negl Trop Dis* 4(10):e856.

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The authors declare no conflict of interest.

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