Author’s response to reviews

Title: Refractoriness of hepatitis C virus internal ribosome entry site to processing by Dicer in vivo

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Author’s response to reviews: see over
Reviewer's report

Title: Refractoriness of Hepatitis C Virus internal ribosome entry site to processing by Dicer in vivo

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Reviewer's report:

Ouellet et al. provided strong evidence in this paper that Dicer can process HCV IRES in vitro but not in vivo. The findings are interesting and they contribute important new facts and thoughts to the field. The idea that the highly-structured HCV IRES can evade cellular RNAi silencing, particularly Dicer processing, not only challenges existing models but might also have broad implications in the study of other viral RNAs. This reviewer enthusiastically recommends the paper for publication in the journal. The following comments and suggestions are just for authors' consideration during the revision.

1) Fig. 4: It might be helpful to add a positive control (e.g. endogenous miRNA) for the RIP experiment.

2) Fig. 4: It might be helpful to express tagged-Dicer in the cells.

3) Although Dicer does not bind HCV IRES in vitro (Fig. 4) and Dicer only processes HCV IRES in vitro, it will be of interest to see whether Dicer can process the "cell-experienced" IRES RNA. For example, after RNA transfection, biotinylated P32-labeled IRES RNA might be isolated and checked for processing by recombinant Dicer (similar to Fig. 2). This might help explain whether the protein complexes associated with IRES might contribute to the resistance to Dicer processing.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.
Response to the Reviewer’s comments

Comment 1: In Fig. 4, it might be helpful to add a positive control (e.g. endogenous miRNA) for the RIP experiment.

Response: We thank the Reviewer for this suggestion, which is interesting. However, considering the high processivity of Dicer in vivo, i.e. the fact that endogenous miRNA precursors (pre-miRNAs) are rapidly converted by Dicer into miRNAs, which are then rapidly transferred to the miRNP complex containing Argonaute 2 (Ago2), may explain our inability to detect endogenous pre-miRNA or miRNA species associated with Dicer immunoprecipitates (IP) analyzed either by Northern blot or more sensitive RNase protection assays (data not shown). Therefore, detection of endogenous miRNAs may not represent an appropriate positive control for the RIP experiment.

Comment 2: In Fig. 4, it might be helpful to express tagged-Dicer in the cells.

Response: The Dicer antibody that we have used in Figure 4 has been characterized previously and shown to recognize and induce a supershift of substrate-bound Dicer complexes (see Provost et al., 2002), thereby supporting its suitability for the RIP experiment. Because immunoprecipitation of endogenous Dicer from Huh-7 and 9-13 cells is rather efficient and that the transfection efficiency of these cells is relatively low (~5 to 10%), we did not use epitope-tagged Dicer expression in the experimental design of Figure 4.

Comment 3: Although Dicer does not bind HCV IRES in vitro (Fig. 4) and Dicer only processes HCV IRES in vitro, it will be of interest to see whether Dicer can process the “cell-experienced” IRES RNA. For example, after RNA transfection, biotinylated P32-labeled IRES RNA might be isolated and checked for processing by recombinant Dicer (similar to Fig. 2). This might help explain whether the protein complexes associated with IRES might contribute to the resistance to Dicer processing.

Response: We presume that the Reviewer intended to mean “Although Dicer does not bind HCV IRES in vivo (Fig. 4) ...”. The proposed experimental strategy is interesting, although it may be limited by the folding and integrity of the in vitro transcribed IRES upon transfection into cells, whose efficiency is relatively low, as mentioned above. In addition, this approach does not alleviate the limitations associated with the (inhibitory effects of) cellular proteins capable of binding the IRES RNA structure.

We assessed “cell-experienced” IRES RNA function in IRES-driven reporter gene activity assays (see Figure 5). In these experiments, we hypothesized that IRES processing by Dicer would decrease its ability to initiate translation. However, we observed that the increased level of Dicer expression did not compromise the
functional integrity of the HCV IRES, suggesting the maintenance of its structural integrity and implying that the refractoriness of the HCV IRES to Dicer processing depend on the cellular environment. Further investigations are needed to clarify this issue (eg, siRNA– or shRNA–mediated knockdown of cellular IRES–binding proteins) and identify the cellular components involved in protecting the HCV IRES from Dicer processing.