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Questioning antiviral RNAi in mammals

To the Editor — I wish to comment on the central claim made in the article by Li *et al.,* which suggests that mammals elicit a small RNA-mediated response to RNA virus infection in somatic cells[1]. While our evolutionary past certainly suggests that our ancestors used RNA interference (RNAi) to limit virus replication, with the emergence of vertebrates came a protein-based defence that hinged on the family of interferons (IFNs)[2]. A question that remains is whether RNAi still represents a physiological component of our cellular antiviral arsenal that works alongside the IFN system.

Over the past three years, four papers (two from Maillard *et al.* and two from Li *et al.*) have reported on observations that provide some support for the idea that RNAi contributes to the mammalian antiviral arsenal[1,3–5]. In 2013, Maillard *et al.* published a study that suggested that pluripotent stem cells retained the capacity to elicit an RNAi response[3]. This claim supported earlier work that found, unlike somatic cells, multipotent lineages such as stem cells could process double-stranded RNA (dsRNA) into small interfering RNAs (siRNAs)[6,7]. In a second paper published back-to-back with Maillard *et al.*, Li *et al.* demonstrated an inverse correlation between the appearance of viral small RNAs and virus replication in suckling mice[5]. While not addressed directly, this later study suggested that RNAi could also be detected in somatic cells, although this seemingly contradicted the work by Maillard *et al.*, which showed RNAi was lost in differentiated cells[8]. More recently, a follow-up paper by Maillard *et al.* demonstrated that RNAi could be observed in differentiated cells only when they were void of a canonical IFN response[4]. As both stem cells and newborn mice have deficiencies in IFN, it seemed that a consensus for the longstanding controversy concerning RNAi in mammals would finally be reached — RNAi in mammals may be observable but only in small subpopulations of cells that are IFN-nonresponsive. However, even this idea, which itself still requires independent corroboration, has now been called into question with the most recent conclusions of Li *et al.* suggesting that mammalian RNAi is readily detectable in IFN-competent somatic cells[1].

The suggestion that RNAi is a *bona fide* component of the mammalian antiviral response in differentiated cells not only conflicts with the publications of many independent groups[9–12], it is also based on data that lack the necessary rigor to make this claim. The 2016 Li *et al.* article begins with findings demonstrating that less than 1% of small RNAs that associate with Argonaute 2 (AGO2) align to an engineered influenza A virus lacking non-structural protein 1 (NS1), a dsRNA binding protein implicated in blocking both IFN[13] and RNAi[14–16]. The authors demonstrate that the virus-derived small interfering RNAs (vsiRNAs) have an RNase III signature and are only produced in the absence of NS1. While provocative, these data are not sufficient to support the claim that RNAi is functional in mammals. Indeed, given that both human RNase III nucleases, Dicer and Drosha, reside in the cytoplasm upon virus infection, one would predict a low yield of these characteristic small RNAs[17–19]. Moreover, the central claim of this work hinges not on the appearance of these small RNAs, but on their functionality. Given this, what is required to prove an intact RNAi response in mammals would be a reporter system that could be silenced by these identified small RNAs in a sequence-specific manner. In the absence of demonstrating any specific silencing activity, the authors correlate their biochemistry data to independent work produced in catalytically inactive AGO2 fibroblasts. Confusingly, the authors use both wild-type influenza virus and a recombinant strain lacking NS1 for this part of their study. As shown in Figs 2 and 3 of the article, wild-type influenza viruses do not generate detectable vsiRNAs and therefore the observed enhancement of replication in catalytically inactive AGO2 fibroblasts does not support a claim for RNAi in mammals. Rather, this data would indicate an indirect effect that enables increased replication, a phenotype that also extends to their encephalomyocarditis virus and vesicular stomatitis virus infections. This interpretation would also explain why Maillard *et al.* failed to see enhanced influenza A virus replication, regardless of NS1 status, in cells lacking AGO2 (ref. 4). While it could be argued that the complete loss of AGO2 is itself an imperfect model given AGO2's influence on microRNA (miRNA) biology[20], the Maillard *et al.* data are consistent with recent findings that unrelated perturbations of miRNA activity have been shown to have little impact on RNA virus replication directly[10,21].

In addition to the aforementioned issue, many independent studies suggest that the central claim of the Li *et al.* article, which states that mammals have retained a functional antiviral RNAi pathway, is incorrect. Independent of the work of Maillard *et al.*, Girardi *et al.* and Seo *et al.* also demonstrated an inherent conflict between the IFN and RNAi systems[9,22]. Attempts to reconstitute an RNAi response in mammalian cells by Girardi *et al.* led to activation of the dsRNA protein kinase R, one of the main effectors of the IFN system that inhibits translation[22]. In the Seo *et al.* study, the authors found that IFN and virus infection resulted in the loss of RNA-induced silencing complex function[9]. Therefore, even in the event of the accumulation of vsiRNAs, their ability to effectively inhibit virus replication would be negligible. Together, these works strongly support the idea that IFN and RNAi, should they ever co-exist, would be functionally incompatible. Moreover, work from my own group and others has found that virus-derived NS1 does not inhibit small RNA-mediated silencing in mammals[11,21,23–27]. One striking example of this can be gleaned from the observation that influenza A virus can be engineered to generate a Dicer-dependent, AGO2-loaded small RNA capable of silencing its own transcript, even in the presence of wild-type NS1[28]. Another observation in conflict with the idea that NS1 antagonizes a mammalian RNAi response comes from the initial work describing the very virus used in the Li *et al.* study. In that work, influenza A virus lacking NS1 retained the capacity to kill mice in the absence of a functional IFN system[13]. Should RNAi function as Li *et al.* suggest, one would assume it would provide protection against a virus lacking its central antiviral antagonist.

Given the ongoing debate concerning the utility of RNAi as a *bona fide* antiviral system in mammals, a statement by one of the greatest philosophers of science, Karl Popper, seems fitting. He stated that the method of science is a method of bold

conjectures and ingenious and severe attempts to refute them[29]. When applied to the concept of mammalian RNAi, the findings of Li *et al.* may yet prove correct, but the burden of proof should remain high given the vast amount of data refuting this idea. It is my opinion that this burden has not yet been met and the question as to whether RNAi represents a physiological component of our cellular antiviral arsenal that works alongside the IFN system remains unanswered. ❐

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Competing interests

The author declares no competing financial interests.

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