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Reply to 'Questioning antiviral RNAi in mammals'

Kate L. Jeffrey[1], **Yang Li**[2], and **Shou-wei Ding**[3]

[1]Gastrointestinal Unit and Center for the Study of Inflammatory Bowel Disease, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114, USA

[2]State Key Laboratory of Genetic Engineering, Collaborative Innovation Centre of Genetics and Development, School of Life Sciences, Fudan University, Shanghai 200438, China

[3]Department of Plant Pathology & Microbiology, and Institute for Integrative Genome Biology, University of California, Riverside, California 92521, USA

Jeffrey *et al.* reply

Benjamin tenOever purports to comment on our claim that "mammals elicit a small RNA-mediated response to RNA virus infection in somatic cells". Our article[1] is a follow-up of two published papers in 2013, which provided the first evidence for an antiviral function of RNA interference (RNAi) in mammals[2,3]. The 2013 studies demonstrated production of canonical virus-derived small interfering RNAs (siRNAs) in suckling mice and cultured mouse embryonic stem cells (mESCs) and hamster cells following infection with positive-strand RNA viruses. Production of the viral siRNAs in all three host systems was strongly inhibited by the B2 protein of Nodamura virus (NoV), known previously to suppress antiviral RNAi in insect cells and siRNA-induced RNAi in mammalian cells[4–6]. Notably, the suppressor activity of B2 is required for NoV infection in all three systems and deletion of Argonaute 2 (AGO2) in mESCs enhanced accumulation of the B2-deletion mutant of NoV significantly more than wild-type NoV, indicating B2 suppression of an AGO2-dependent antiviral RNAi mechanism in mammalian cells[2,3]. However, many questions remain to be addressed in mammalian antiviral RNAi. Our new study aimed firstly to understand why many previous deep sequencing studies were unable to detect viral siRNAs in mature human somatic cells infected with a range of RNA viruses. These unsuccessful attempts, including one by tenOever and colleagues, to deep sequence small RNAs from human A549 cells infected with wild-type A/Puerto Rico/8/1934(H1N1, PR8) strain of influenza A virus (IAV)[7] have led to the idea that the conserved machinery of RNAi is unable to detect RNA virus infection in interferon (IFN)-competent mammalian somatic cells.

Our study[1] has shown that human HEK-293T, A549 cells and monkey Vero cells produce highly abundant viral siRNAs after infection with either the same PR8 strain of IAV or a related WSN strain, A/WASN/1933(H1N1). Two technical improvements were critical for our success. Firstly, host cells needed to be infected with a mutant IAV lacking function of the viral non-structural protein 1 (NS1), known to suppress antiviral RNAi in insect cells and

Competing interests

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siRNA-induced RNAi in mammalian cells[4,8]. Secondly, the small RNAs that are not specifically associated with AGOs and the RNA-induced silencing complex (RISC) needed to be removed by only including AGO co-immunoprecipitation RNAs into the construction of small RNA libraries for sequencing. Using this approach, the relative abundance of influenza viral siRNAs in the mature human somatic cells is comparable to those found in mESCs[3] and *Drosophila* cells[9]. Notably, single species positive- and negative-strand influenza viral siRNAs are readily detectable by northern blot hybridization using regular RNA probes[1], a key criteria used in microRNA (miRNA) annotation[10]. We demonstrated that the influenza viral siRNAs become undetectable by either deep sequencing or northern blotting in Dicer knockout cells, and that the defect in the viral siRNA biogenesis was restored by ectopic expression of human Dicer. *Drosophila* Dicer-2, but not Dicer-1, which are responsible for the biogenesis of viral siRNAs and cellular miRNAs, respectively[11], could also produce influenza viral siRNAs in human cells, although the predominant size shifts from 22 nucleotides (nt), made by human Dicer, to 21 nt by *Drosophila* Dicer-2 (ref. 1). Moreover, the sequenced influenza viral siRNAs are highly enriched for the canonical siRNA duplexes with 2-nt 3′-overhangs[1], supporting the proposed model in which viral siRNAs are produced by human Dicer that uses double-stranded RNA (dsRNA) viral replicative intermediates as precursors. Consistent with the biogenesis of mammalian miRNAs by the same Dicer enzyme, Dicer-produced influenza viral siRNAs are abundantly loaded into AGOs and exhibit strong preference for uracil as the 5′-terminal nucleotide[1].

Our findings explain why tenOever and colleagues failed to detect the influenza viral siRNAs in human cells infected with NS1-expressing wild-type IAV[7]. Indeed, ectopic expression of NS1 can suppress production of siRNAs from either artificial long dsRNA[12] or influenza viral dsRNA replicative intermediates[1]. We have also detected abundant virus-specific small RNAs not associated with RISC in infected human cells, which must be removed before the canonical properties of the viral siRNAs are visible[1]. We predict that AGO co-immunoprecipitation would improve the detection of viral siRNAs from viruses that do not have a suppressor to inhibit siRNA biogenesis.

We did not develop a reporter system for the influenza viral siRNAs, frequently used to assay for the activity of a miRNA or siRNA to target a steadily transcribed messenger RNA. Instead, we used a genetic approach commonly employed to assess the impact of antiviral RNAi directly by comparing virus titres between wild-type and RNAi-defective mutant cells[3,11,13–19]. The primary mouse embryonic fibroblasts (MEFs) carrying a genetic mutation ($Ago2^{D597A}$) that abolishes the activity of RISC programmed by siRNA to slice target RNA[20] are the only available RNAi-defective mature somatic mammalian cells that are IFN-competent and exhibit no known defect in miRNA function[20]. Our results illustrate that three distinct RNA viruses replicate to significantly enhanced levels in the RNAi-defective mutant MEFs when compared to wild-type MEFs[1]. Our findings are consistent with the observation that RNAi-defective fruit flies are much more susceptible than wild-type flies to all viruses examined, including those expressing proteins that potently, but incompletely, suppress RNAi[14–16]. In the comments[21] made on Benitez *et al.*'s article[22] and related studies, pioneering work to engineer expression of miRNAs from viral RNA genomes[23,24] have been presented and discussed to explain why these recombinant viruses remain susceptible to the artificial miRNAs despite expression of potent RNAi suppressors.

Notably, abolishing the catalytic activity of AGO2 in MEFs is significantly more effective to enhance accumulation of the NS1-deletion mutant of IAV (delNS1) than wild-type IAV[1], providing strong physiological evidence for both the induction and suppression of antiviral RNAi in mature mammalian somatic cells.

One of the important questions that remain to be fully investigated is the relative contribution of antiviral RNAi to mammalian antiviral immunity. To this end, we have shown that induction of IFN-stimulated genes (ISGs) is similar in wild-type and RNAi-defective MEFs[1]. Suppression of RNAi by the B2 protein of NoV also does not alter the expression of ISGs in infected mice[2]. Furthermore, production of abundant influenza viral siRNAs is readily detectable in both Vero cells and A549 cells that are defective and competent in the IFN system, respectively[1]. Importantly, abolishing the catalytic activity of AGO2 further enhances virus titres in MEFs when IFN signalling is blocked, indicating that antiviral RNAi restricts virus infection independently of IFN signalling[1]. Adolfo García-Sastre, Peter Palese and colleagues have shown previously that the delNS1 virus grows to one-log-lower titres in Vero cells and to one-to-two-log-lower titres in the lung of *STAT1*$^{-/-}$ (*Signal transducer and activator of transcription 1*) mice (defective in IFN signalling) when compared to wild-type IAV[25]. This suggests that an antiviral mechanism independent of IFN signalling, which in principle could include antiviral RNAi, is suppressed by the multifunctional NS1 (ref. 26).

A multitude of mammalian host defence systems have evolved against viral pathogens and their goal is to interfere with viral replication and propagation or to kill infected cells. These systems cooperate, complement or compensate for each other to allow for greater coverage of the microbial world and robustness in the face of continuous pathogen evolution[27]. Indeed, the IFN antiviral system acts to regulate many distinct effector mechanisms, and much evidence now suggests that this includes antiviral RNAi[28,29]. Similarly, there is evidence that RNAi components regulate pathways that lead to IFN production[30,31]. Our data demonstrate that in mature mammalian cells, antiviral RNAi is active either in the presence or absence the IFN system. The respective contribution of IFN-regulated antiviral systems and RNAi in diverse cell types with variable IFN-regulated cell responses or Dicer activity, or against the many viruses that possess RNAi and IFN suppressor proteins to achieve optimal defence against rapidly evolving viruses, will be the subject of many future scientific studies.

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