Chapter 13

RNAi-Induced Immunity

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Abstract

RNA interference has a close relationship with the host defense system including adaptive immunity. It is not only involved in regulating immune cells at different stages of the immune response but also directly induces or enhances antigen presentation and subsequent immune responses. We have previously reported that a small hairpin RNA (shRNA) targeted the downstream site of a dominant cytotoxic T lymphocyte (CTL) epitope of human papillomavirus (HPV) type 16 oncogene E7 can stimulate an immune response against E7 expressing tumors in C57BL/6 mice. This results in the elimination of tumor growth *in vivo*, whereas an shRNA that targets the upstream site does not. Our recent data further confirm the long half-life of the 5'-mRNA fragment after shRNA degradation and its involvement in protein synthesis. This chapter summarizes these findings and provides some updated explanations for the findings.

Keywords: RNAi, shRNA, immune response, HPV E7, miRNA

1. Introduction

RNA interference (RNAi) is a conserved gene-regulation mechanism in all eukaryotic cells, where small RNAs including small interfering RNA (siRNA), small hairpin RNA (shRNA), and micro-RNA (miRNA, miR) interact with message RNAs (mRNAs) in a sequence-specific manner and cause the cleavage or translational blockage of a gene [1, 2]. Because of its specificity and efficiency, it has been widely utilized as a routine tool for gene functional studies in biology laboratories worldwide. In addition, since RNAi blockage is very specific and at the transcriptional level, RNAi-based gene therapy (RNAi therapy) is thought to hold a great potential for treating many diseases, especially viral infections and genetic disorders. Synthesized siRNA is thus regarded as a specialized drug for gene therapies. So far, promising results have been obtained with RNAi therapy in various diseases and many are being tested in clinical trials, including viral infections, cancers, and genetic or inflammatory dis-

orders [3-7]. As cancers and other emerging diseases such as dementia and super-bug infections become major public health issues, RNAi therapy can offer a new solution and has the additional ability to overcome drug resistances.

Beside the fundamental gene-silencing and gene-regulating roles of RNAi, which will also regulate the gene functions of immune cells and thus the immune responses, RNAi pathway itself has an additional function and involvement in inducing adaptive immunity. This function has not been well-studied, and the mechanism is not clear. Its relationship with the immune system in terms of antigen reactivation and antigen presentation is still a new area to be investigated. Indeed, in plants and primitive species, RNAi is a part of the defense system against viral infections. However, in mammals, RNAi seems not directly involved in the immune system, probably due to the development of an advanced and sophisticated immune system. This chapter summarizes the evidence of RNAi-induced immunity against tumors and provides some updated possible explanations for the findings. The possible link between miRNA and its degraded products with the immune system has been also discussed. Exploring the relationship between RNAi and the immune system may lead to new discoveries in RNAi biology and approaches for more effective cancer immunotherapy or treatment for viral and intracellular pathogen infections.

2. The discovery of RNAi-induced adaptive immunity against tumors

In 2009, we reported a discovery about RNAi-induced immunity [7]. We investigated two shRNAs encoded by lentiviral vectors on their ability to suppress tumor cell growth and stimulate antitumor immunity *in vivo*. One shRNA targeted the downstream site of a dominant cytotoxic T lymphocyte (CTL) epitope of the oncogene E7 of human papillomavirus (HPV) type 16 (termed downstream shRNA), while another shRNA targeted the upstream site of this epitope (termed upstream shRNA). Both shRNAs were equally effective at silencing E7 gene expression (in mRNA and protein levels) and led to the inhibition of tumor cells growth *in vitro* and *in vivo* [7]. In spite of this, TC1 tumor cells (expressing HPV E6 and E7) treated with downstream shRNA stimulated an immune response against E7 in C57BL6 mice and resulted in elimination of tumor growth *in vivo*, whereas cells treated with the upstream shRNA did not. When untreated TC1 tumor cells were injected to the same mice (challenging tumors), the group of downstream shRNA exhibited a total inhibition of challenging tumor growth, whereas no inhibition was observed in the upstream shRNA group. This ability of downstream shRNA was absent in $Rag^{-/-}$ mice (lack of T- and B-cells), suggesting adaptive immune response or T-cell response was required. To prove that the immune response was antigen-specific, we carried out a same animal experiment by immunizing C57BL/6 mice with TC-1 cells treated with these shRNAs but challenged with another tumor cell line C2, which has the E7 expression and H-2b genetic background as C57BL/6 mice. Again, we observed that only mice immunized with downstream shRNA treated cells had a loss of tumor formation, indicating tumor clearance was specific to E7. Our data indicate that a more effective treatment can be developed for cervical cancer by combining RNAi

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treatment with immunotherapy. Our results also reveal that RNAi may be widely used as an antitumor immunity stimulator or enhancer (Fig 1).

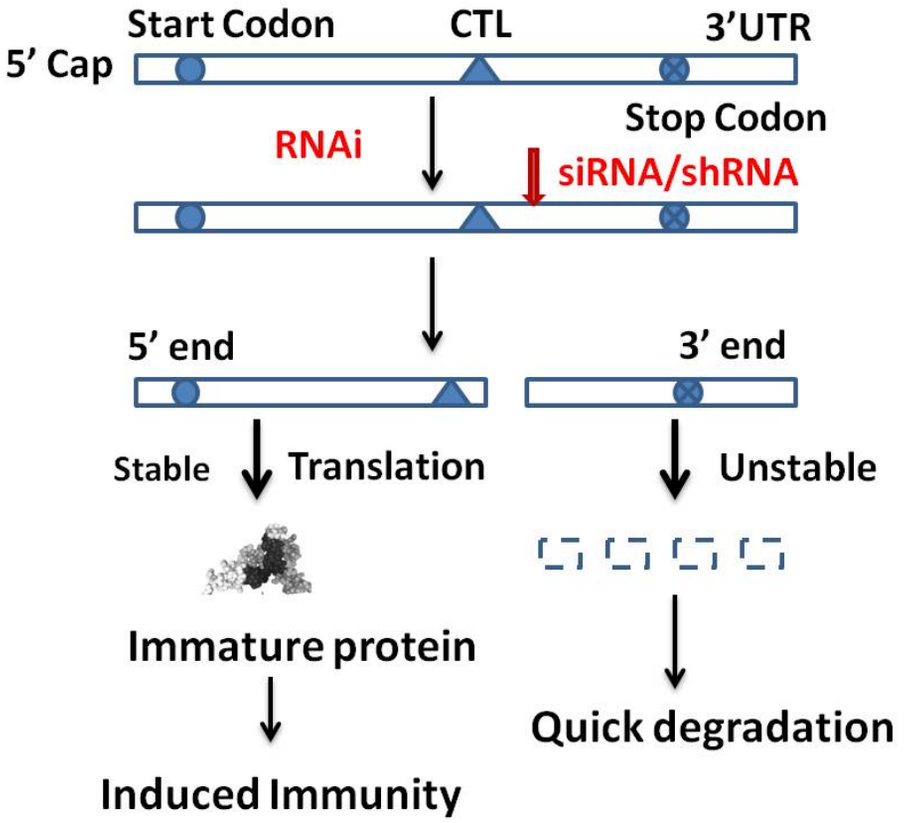

Figure 1. Schematic diagram shows RNAi targeting site and 5' mRNA fragment. It is known that in cervical cancer HPV E7 and mouse lymphoma EG7/OVA (ovalbumin) models, the shRNA targets the downstream site of the CTL epitope that produce the 5' fragment of mRNA. This makes immature proteins and further induces an immune response.

To prove the applicability of immune response to general tumor antigens, we tested a model antigen ovalbumin (OVA) expressed in EG7 cells that are a mouse thymoma cell line with C57BL6 genetic background. We chose the major CTL epitope of OVA, SIINFEKL, as the target site and designed two upstream shRNAs OVA-1 and -2 and a downstream shRNA OVA-3. The shRNA-treated EG7 cells were used to immunize the mice, which were subsequently challenged with untreated EG7 cells. We observed that only mice inoculated with OVA-3-shRNA treated cells had significantly reduced tumor formation but not with OVA-1 and -2 shRNAs.

3. Confirmation of mRNA fragments and truncated proteins in treated cells

To determine if the RNAi-induced immune response was actually from degraded products of RNAi, we designed a series of primers that would amplify RNA fragments inside and

outside the targeting sites of upstream shRNA (E6-1) and downstream shRNA (E7-1, Fig 2A). If an inside fragment was present while the outside fragment was absent, it would indicate that the mRNA had been cleaved by shRNA at the target site. The cells were treated with E6-1 and E7-1 shRNAs and incubated for 2–4 days before real-time RT-PCR was carried out. As expected, the shortest PCR fragment, R3, was observed in all samples (Fig 2B). The full-length R1 PCR fragment was only observed in untreated TC1 cells or cells infected with the lentiviral vector control (PLL, Fig 2B). In cells treated with both E7-1 and E6-1, R1 was not found, indicating that shRNA-mediated cleavage was occurring. Of most interest was the R2 fragment which was found in all samples except cells treated with E6-1. These results suggest that R2 or R3 short fragments of E6-1 and E7-1 existed in the cells, at least temporarily at the time we isolated RNA. These short-form mRNAs may act as templates for short-form proteins (truncated proteins) and trigger antigen presentation to CD8+ T-cells and a CTL immune response to E7.

Apart from our data, a previous study reported that degradation of the 3' mRNA fragment resulting from siRNA-mediated cleavage was blocked for some mRNAs, leaving an mRNA fragment that could act as a template for cDNA synthesis. They suggested that this could give rise to false negative results and that this phenomenon may be avoided by the careful design of RT-qPCR primers for each individual siRNA experiment [8]. This report further confirms that mRNA fragments from RNAi do sometimes exist in the cells. In addition, it was noticed by researchers that un-degraded fragments of an siRNA-targeted mRNA may cause false positive effects of microarray analysis [9]. To avoid this, they developed a qRT-PCR protocol, which allowed for the determination of the optimal time point for mRNA analyses, indicating mRNA fragments after RNAi can be present in cell for a certain time.

What is the functional role of these mRNA fragments after RNAi? Our data demonstrated that they can be involved in translational machinery and produce truncated proteins. To experimentally prove this, we utilized the OVA-expressing EG7 cell model again. The cells were treated with downstream and upstream shRNAs and further treated with the protease inhibitor MG132 to reduce protein degradation before immunoblotting was performed. The blots were probed with an antibody against the N terminus of OVA protein. The predicted size of a truncated protein produced by the cleavage of OVA-2 shRNA was 14.7 kDa. We observed a protein band about 15-kDa in cells treated with the OVA-2 shRNA but not in untreated and OVA-1 or OVA-3 shRNA treated cells. It proves that truncated proteins can be produced in cells by the translation of mRNA fragment cleaved by shRNA. The predicted truncated product by shRNA-OVA3 was not observed due to cross-reacting proteins on the blot [7].

Our recent data (unpublished) showed that the cleaved 5′ and 3′ fragments of human papillomavirus type 16 (HPV-16) E6/7 mRNA after shRNA treatment were unevenly degraded. The 5′ mRNA fragment was more abundant and displayed a greater stability than the corresponding 3′ fragment in the treated cells. Further analysis revealed that the 5′ fragment was polysome-associated, indicating its active translation, and this was further confirmed by using tagged E7 protein to show that C-terminally truncated proteins were produced in treated cells (Singhania et al. submitted).

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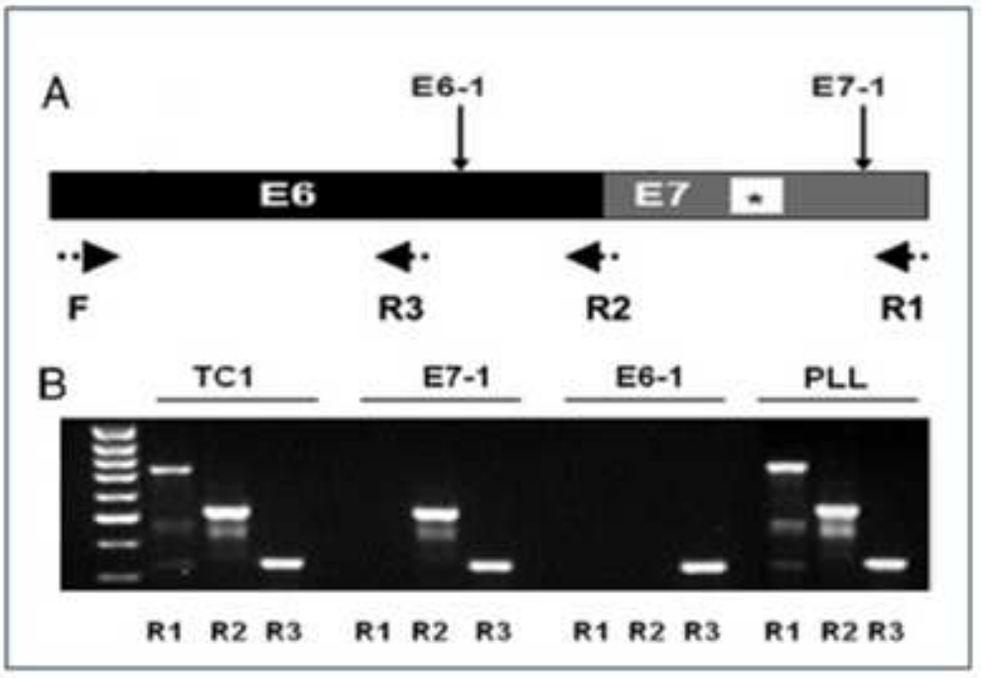

Figure 2. The shRNA targeting sites and primer design for HPV E6/E7 mRNA (Adapted from Gu et al 2009). (A) The shRNA and primer sites on E6/E7 mRNA. (B) The PCR products on an agarose gel. Notes: TC1, the untreated cell control. PLL, lentiviral vector control. *: indicates the site of CTL epitope. F: forward primer, R: reverse primer.

4. Possible models for explaining RNAi-induced immunity

It is well established that miRNAs play an important role in regulating innate and adaptive immune responses as a part of their gene-regulating roles. Evidence has accumulated that miRNAs are involved in the adaptive immune responses by regulating T-cells, B-cells, and antigen-presenting cells (APCs). For example, miR-214 was reported to target phosphatase and tensin homolog (PTEN) and increase proliferation and the activation of T-cells [10], while miR-150, -155, and let-7 have been shown to be involved in the development of T-cells into memory cells [11]. In addition, miR-184 was shown to inhibit nuclear factor of activated T-cells-1 (NFAT-1) in the activation of CD4 T-cell in the early stage of adaptive immune responses [12] and miR-181-a could promote CD4 and CD8 double positive T-cell development [13]. For B-cells, miR-155 is required for their normal function such as production of isotype-switched, high-affinity antibodies and for memory responses [14]. It has also been demonstrated that miR-155 is induced by B-cell receptor (BCR) [15]. However, overexpression of miR-155 can immortalize B-cells and lead to transformation, for instance, EBV was shown to have induced miR-155 expression and transformed B-cells [16, 17]. In addition, miR-150 is important in B-cell development [18] and so is miR-17 [19, 20].

For dendritic cells (DCs), a recent review summarized the need of miRNAs in their lineage commitment from bone marrow progenitors and for the development of subsets such as

plasmacytoid DCs and conventional DCs [21]. Liu et al. (2010) used software to predict and then conducted experiments to confirm that three members of the miR-148 family, miR-148a, miR-148b, and miR-152 are negative regulators of the innate immune response and antigen-presenting capacity of DCs. They showed that miR-148/152 expression was upregulated in DCs on maturation and activation induced by TLR3, TLR4, and TLR9 agonists. These miRNAs in turn inhibited the production of cytokines including IL-12, IL-6, TNF-alpha, and IFN-beta and upregulation of MHC class II expression and DC-initiated antigen-specific T-cell proliferation by targeting Calcium/calmodulin-dependent protein kinase II alpha (CaMKIIα) [22]. In addition, miR-150 and miR-223 has been shown to play an important regulatory role in Langerhans cells (LCs) by cross-presenting a soluble antigen to antigen-specific CD8(+) T-cells [23, 24]. Beside APCs such as DC and LC, miRNA is shown to be directly involved in antigen presentation. For example, Bartoszewski et al. (2011) demonstrated that the mRNA of human endoplasmic reticulum (ER) antigen peptide transporter 1 (TAP1) is a direct target of miR-346. They showed that the 3'-UTR (un-translational region) of TAP1 contains a 6-mer seeding region for miR-346 and the ER stress-associated reduction of TAP1 mRNA and protein levels could be reversed by inhibitory miRNA of miR-346 [25]. As TAP plays an important role in MHC class I-associated antigen presentation, their data provide an insight for miRNA-regulating MHC class-I-associated antigen presentation during ER stress.

The above-highlighted results clearly indicate miRNA's regulatory role in many aspects of adaptive immunity. However, is it possible that miRNA also takes part in host immunity through mRNA fragments produced after RNAi just like shRNA described in section 2 and 3? Normally, miRNAs target the 3' UTR and lead to the translation block or degradation of the targeted mRNAs. So when they degrade mRNAs, it is supposed to produce long 5' mRNA fragments and may also produce truncated proteins. If this is true, the translated defective proteins could be treated as truncated protein and be processed by proteasome. If there is a CTL epitope in the defective structure, it could be coupled with the MHC class I molecule and presented to T-cells by DCs through antigen cross-presentation, as described above with shRNA. This could be a link between RNAi pathway and antigen presentation or adaptive immune response.

In the shRNA case discussed above, the target should be at the downstream of a CTL epitope to induce immunity. When miRNAs target 3' UTR, a site certainly at the downstream site of any possible CTL epitopes, it is assumed to have the ability to produce truncated proteins and so to induce immune responses. Therefore, an important question for miRNA biology is whether miRNAs can routinely induce immune responses by degrading mRNA at 3' UTR and generating 5' mRNA fragments or truncated proteins that contain CTL epitopes? So far, there is no answer for this question. Another critical question is: what is the difference between blocking and degrading mRNA by miRNAs at 3' UTR? Does this relate to antigen presentation of different proteins?

Because it has been shown that miRNA can act as siRNA and shRNA can be produced in the same pathway as miRNA [26], it is important and interesting to investigate if miRNA can induce the same immune response as shRNA. The systems of HPV 16 E7/TC1 and

OVA/EG7 can be used as good models to investigate this. As miRNAs are routinely transcribed and involved in interacting with mRNA, this mechanism can be considered as a routine way in cells to generate CTL containing truncated proteins. However, because most mRNAs in cells are for self-proteins, their CTL epitopes will not be presented to T-cells. This leaves the question of whether this is a mechanism just for cells that express viral genes (such as HPV E7 in TC1 and C2 as above) or for cells expressing foreign genes/antigens (such as OVA in EG7)? The next question is: can this be generalized to any tumor antigens including self-antigens? This is an interesting subject to investigate and will facilitate our understanding of how RNAi pathways interact with and are involved in adaptive immune responses (antigen presentation) to utilize them for cancer immunotherapy.

Although some miRNA are highly conserved between lower animals and higher animals, mammals have far more miRNAs compared to nonmammals. This suggests that during evolution, as gene regulation became so complex and important in higher animals, miRNA or RNAi pathway gradually specialized into gene regulation. At the same time, as the adaptive immune system became well developed and highly specialized, these two systems got separated, but as described above, they still have some links. Future investigations leading to insight into these links will provide answers to the above questions.

5. Conclusion

In summary, RNAi-induced immunity opens a new perspective in which to explore the relationship between RNAi pathways and the immune system, especially its involvement in antigen presentation in the adaptive immune response. For RNAi biology, it will provide an insight into the understanding of function roles of RNAi (including miRNA and siRNA) in host defense. In the field of gene therapy for cancers, RNAi can be used as an approach to silence oncogenes as well as a strategy to enhance immunity against cancer antigens (at least viral infection related cancers) and further explored as a novel cancer immunotherapy. Finally, for intracellar pathogens, it can be used as a strategy for developing new vaccine through RNAi reactivating their antigens to the immune system.

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