RIG-ing an antitumor response

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A small interfering RNA has been engineered to silence an oncogene and activate the immune response simultaneously. The approach shrinks tumors in mice (pages 1256–1263).

Although it is only seven years since RNA interference (RNAi) was shown in mammalian cells, efforts to harness this powerful and specific gene silencing mechanism to treat human illness by knocking down expression of disease-causing genes have proceeded with lightning speed[1]. Already, a phase 3 study to treat age-related macular degeneration and several phase 2 studies are in progress.

Although a high degree of sequence homology is required to trigger the cleavage of a target messenger RNA, unintended effects on expression of nontargeted genes can occur either because of partial complementarity to a short stretch of as few as seven nucleotides or by triggering immune sensors of foreign RNA that alert the host to viral infection[2]. Because these off-target effects interfere with the potential exquisite sensitivity of small interfering RNAs (siRNAs) to silence one gene at a time, almost as soon as an off-target phenomenon has been described, potential solutions to avoid it have been found, mostly by engineering chemical modifications that maintain silencing activity but abrogate off-target effects[3].

Now, Poeck *et al.*[4] have turned the disadvantage of off-target immune stimulation on its head. They have designed a small RNA, acting as an antitumor agent to not only silence the antiapoptotic gene *Bcl2* but also to bind and activate retinoic acid-inducible gene-I (RIG-I), a cytosolic sensor of viral RNA[5] (**Fig. 1**). By silencing *Bcl2*, the researchers promoted tumor cell apoptosis, and, by activating RIG-I, they stimulated the immune system to destroy the tumor. The antitumor effect of the dually active molecule in a mouse model of metastatic melanoma to the lung was more potent than small RNAs that just activate RIG-I or just knock down *Bcl2*.

Several classes of immune RNA sensors can potentially be activated by siRNAs. These sensors distinguish self from nonself RNA on the basis of cellular localization, biochemical features or, potentially, by differences in sequence. When they are triggered, they activate transcription factors, including interferon response factors and nuclear factor-κB, that stimulate innate and adaptive immunity. Our understanding of what triggers binding and activation of these immune alarms is still incomplete, making it difficult to know how to avoid them or activate them in a controlled fashion.

The cytosolic RIG-I–like helicases (RLHs) are just one class of RNA immune sensors. Others include the protein kinase receptor that recognizes double-stranded RNAs longer than 30 nucleotides (and can largely be ignored for siRNA triggering), Toll-like receptors expressed on the cell surface or in endosomes and, possibly, the large family of cytosolic Nod-like receptors. These sensors differentially respond to particular viruses.

RIG-I and melanoma differentiation-associated gene-5 (MDA5) are the two known RLHs capable of activating an innate immune response to viruses. The helicase domains of these proteins are structurally similar to the RNAi enzyme Dicer. In the lung, RIG-I, activated in myeloid dendritic cells (DCs) and macrophages, may constitute the primary alarm system, whereas TLR7 and TLR8 in plasmacytoid DCs may serve as a back-up whose activation may have more serious systemic consequences[6]. RIG-I expression activates type I interferon (IFN) expression and is in turn upregulated by type I IFNs, providing a mechanism for feed-forward amplification and potential toxicity.

As tumors grow and metastasize, or during chronic infections, the immune system becomes tolerant—unable to distinguish the transformed or infected cell from itself. One potential way to break immune tolerance is to trigger the danger signals that alert immune cells to action. Poeck *et al.*[4] have accomplished this goal by using siRNAs to activate RIG-I, which in turn mobilized anti-tumor immunity mediated by natural killer cells.

siRNAs with two-nucleotide 3′ overhangs that resemble endogenously processed microRNAs are used for most therapeutic applications. They can bind to RIG-I, but they do not activate its signaling[7]. To generate siRNAs that activate RIG-I, the authors took advantage of its ability to distinguish foreign (or nonself) cytosolic RNAs from host cell mRNAs[2]. RIG-I recognizes the 5′-triphosphate RNA produced by viral polymerases. Triphosphates in the cytosol are recognized as a sign of infection, as the 5′-triphosphate end of cellular mRNAs is modified by capping before nuclear export. The authors replaced the 5′-monophosphate ends of most synthetic siRNAs with 5′-triphosphates (3p-siRNA) [2]. The 5′-triphosphate–modified *Bcl2*-specific siRNA was designed to activate RIG-I and simultaneously knock down *Bcl2*.

In one tumor model (B16 melanoma), both effects seemed to be important in limiting tumor metastases, although in another model the immunostimulatory effect on its own was protective, as a control 3p-siRNA was equally effective as the *Bcl2*-specific 3p-siRNA. Even in the B16 melanoma model, the immunostimulatory effect of the 3p-siRNA seemed to be the dominant mechanism of action, as tumors overexpressing an siRNA-insensitive form of *Bcl2* were suppressed almost as much as wild-type tumors. Moreover, there was no significant protection in mice genetically deficient in the type I IFN receptor. Most systemic IFN-α was produced by DCs, as serum IFN-α abundance dropped dramatically in mice depleted of $CD11c^+$ DCs. However, tumor cells also produced IFN-α, which could have contributed to tumor suppression, either by enhancing cell autonomous apoptosis or by making the tumor cells better targets for NK cell elimination. On the basis of antibody depletion experiments, NK cells were crucial for protection.

As the authors point out, the system would easily be adaptable to targeting other cancer-promoting genes[4]—some of which might produce a stronger antitumor effect than targeting *Bcl2*, given that anti-apoptotic Bcl-2 family members (such as myeloid cell leukemia sequence-1, whose *in vivo* expression was upregulated in one treated tumor) can compensate for Bcl-2 loss and that an antisense oligonucleotide drug targeting *Bcl2* has not lived up to its promise in clinical cancer studies[8].

It is not completely clear which cells were the most important direct targets of the

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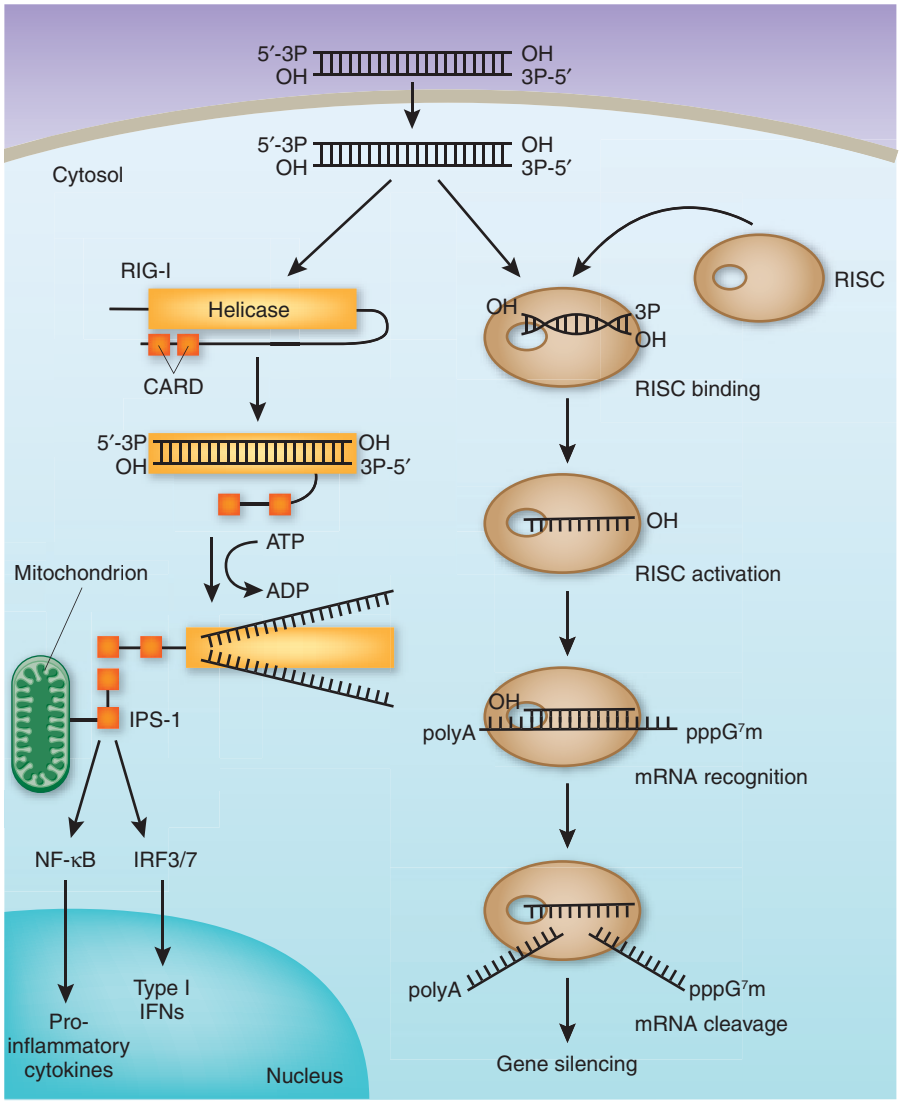

Figure 1 A dual-function small RNA. To produce a single molecule capable of both gene silencing and activating the immune RNA sensor RIG-I, Poeck *et al.*[4] designed a *Bcl2*–specific siRNA with 5′-triphosphate blunt ends (5′-3P) to replace the 5′-monophosphate overhangs used for most siRNAs that mimic Dicer-processed miRNAs. In the cytosol, 3p-siRNA binds to both the helicase domain of RIG-I (left) and Argonaute2 in the RISC (right). The helicase activity of RIG-I activates its CARD domains to bind to the CARD domain of mitochondria-associated IFN-β promoter stimulator-1 (IPS-1), which activates NF-κB, IRF-3 and IRF-7 to translocate to the nucleus to stimulate transcription of proinflammatory cytokines and Type I IFNs, respectively. At the same time, Argonaute2 unwinds the 3p-siRNA and cleaves the sense passenger strand, leaving the antisense strand in an activated RISC that can seek and destroy mRNAs bearing a complementary sequence such as *Bcl2* mRNAs. pppG^{7}m, 7-methylguanosine cap.

immunostimulatory siRNA *in vivo*. Confocal microscopy showed that cells within the tumor took up the siRNA, and, because *Bcl2* was knocked down in sorted tumor cells from treated mice, the siRNA was clearly taken up by the tumor cells themselves. However, it is not certain whether immune cells within the tumor might also have taken it up and been activated to recruit other immune cells into the tumor. Immune activation and IFN production within the tumor, either by the tumor or by infiltrating immune cells, rather than at a distance would be expected to reduce potential systemic toxicity and enhance the local antitumor benefit. Future work to co-stain for IFNs and tumor cell and immune cell markers within the tumors of treated mice could clarify this question.

It remains to be seen whether activation of RIG-I can be controlled enough to be valuable clinically. Turning on a potent type I IFN response that activates a wide range of innate and adaptive immune cells can have serious adverse effects, particularly if the therapy is administered systemically, as would be required for cancer. Mice treated with 3p-siRNA developed lymphopenia, thrombocytopenia and potent systemic activation of myeloid and plasmacytoid DCs, NK cells and T cells. The systemic immunostimulatory siRNA may have adversely affected bone marrow function. Specific delivery of immunostimulatory siRNAs into tumor cells or classes of immune sentinel cells might stimulate antitumor immunity more effectively, with fewer systemic side effects.

Because the RLH and RNA-induced silencing complex (RISC) coexist in the same cytosolic compartment and can both bind the 3p-siRNAs, they may compete for siRNA binding. Activation of the receptor with the higher affinity for the substrate, which is unknown, may dominate in one cell or another. If activating RIG-I turns out to be clinically safe, it might be preferable to separate the gene silencing and immunostimulatory RNAs into two co-administered small molecules.

Moving forward, additional studies are needed to define the protective mechanism of action of the immunostimulatory siRNAs better. As siRNA-based therapies enter clinical testing, it is important to use animal models to follow siRNA uptake and intracellular localization in target cells, as well as in tissue immune phagocytic cells, and clearly show specific gene silencing in the intended target cell, monitor induction of IFN and proinflammatory cytokines, and look carefully for potential toxicity. Understanding the mechanisms of action of the siRNA in animal models will aid in clinical study design to minimize adverse events.

A better understanding of the biophysical characteristics of small RNAs that activate RIG-I, the RIG-I expression pattern and the relative binding affinity of RIG-I versus the RISC will enable researchers to optimize therapeutic approaches.

COMPETING INTERESTS STATEMENT
The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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