Shooting the Messenger: Harnessing RNA Interference to Combat HIV Infection

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IT ALL BEGAN LESS THAN TEN YEARS AGO, WHEN A TEAM OF RESEARCHERS under the direction of Dr. Rich Jorgensen, who is currently an associate professor in the Department of Plant Sciences at the University of Arizona, was experimenting with petunias (Jorgensen, 1996). Dr. Jorgensen's group was attempting to deepen the color of these household plants with the use of a pigment-producing gene. However, upon injecting the plants with the gene, the flowers actually lightened considerably, turning white in some cases. After some sleuthing, Dr. Jorgenson's team suggested that what was being seen was "cosuppression"—the suppression of both the homologous endogenous gene and the introduced pigment-producing gene.

Additional studies conducted over the years have found that this is not simply a phenomenon related to petunias. Gene-expression silencing can occur in a variety of plants, nematodes, Drosophilia, and mammals. "This is basically an ancient mechanism for plants to deal with viral infections," Dr. Judy Lieberman said during her introductory remarks. "Once we saw that it also occurs in mammals, a number of labs set out to determine if it could be used against viruses that infect humans." Over the past few years, there have been a number of papers suggesting that this approach can silence or inhibit numerous viruses, including HIV, human papillomavirus, hepatitis B virus, hepatitis C virus, and others. "At least in tissue cultures," she added, "we know that we can silence these viruses through RNA interference, and there's also potential here in the treatment of cancer and neurodegenerative diseases."

All genetic information in the human body must first be converted to messenger RNA (mRNA) in order to guide the production of proteins—the actual stuff of cellular actions (see Figure 1). The process of RNA interference (RNAi) begins when the enzyme Dicer—a member of the RNase III family of double-stranded RNA (dsRNA)-specific ribonucleases—cleaves dsRNA to form degraded short interfering segments of double-stranded RNA (siRNA) containing approximately 19 base pairs each and approximately 21 to 23 nucleotides in length. From there, siRNAs are assembled into endoribonuclease—containing complexes known as RNA-induced silencing complexes (RISCs). The siRNA strands are then unwound to form activated RISC. The siRNA strands subsequently guide the RISCs to mRNA—cleavage of mRNA takes place near the middle of the region bound by the siRNA strand—ultimately silencing the intended actions of the gene.

Given that RNAi is so specific in the mRNA it targets, it is able to silence the instructions of particular genes while leaving other genes free to go about their business. This has been a breakthrough for those involved in basic science, as it has allowed researchers to focus on the function of particular genes. There is therapeutic potential here as well and there are two central avenues to explore (see Figure 2). The first involves silencing the genes responsible for the expression of key receptors and coreceptors on uninfected cells to prevent viral entry. The second involves the silencing of viral genes responsible for the replication of HIV inside infected cells.

While Dr. Lieberman was quick to point out that it's way too early to categorize RNAi as a *bona fide deus ex machina,* "it is an approach that we do think has potential and we are very excited about it."

Silencing CD4: Proof of Concept

AS A PROOF OF CONCEPT, DR. LIEBERMAN AND HER COLLEAGUES FIRST SET out to investigate the feasibility of using siRNA to halt HIV entry by way of targeting and suppressing the CD4 molecule (Novina, 2003). Her colleagues employed Magi-CCR5 cells, which express human CD4, CXCR4, and CCR5. These cells also contain an integrated gene encoding the β-galactosidase gene which, Dr. Lieberman explained, "cause the cells to turn blue when they are infected with HIV."

The results of this study were encouraging. Approximately 75% of the Magi-CCR5 cells transfected with CD4-siRNA had reduced CD4 expression, which was determined using flow cytometry. Additional experiments using a Northern-blot assay revealed an approximate eightfold reduction in CD4 mRNA. This eightfold reduction in CD4 mRNAs resulted in a fourfold reduction in viral entry measured by β-galactosidase activity. CD4-siRNA-transfected cells also showed few syncytia, which would be associated with limited viral spread to neighboring uninfected cells.

"These were important findings," Dr. Lieberman said. "However, it's not really practical to silence CD4, as it is a very important molecule for immune function."

Silencing Viral Genes: Kicking HIV Where it Counts

VIRTUALLY ALL OF HIV'S GENES CAN BE TARGETED USING siRNA. TO TEST this assumption, Dr. Lieberman's group conducted a preliminary study training siRNA on the gag gene, which plays a central role in viral genomic RNA accumulation and production of p24 (Novina, 2003).

First off, Dr. Lieberman's team transfected CD4+ cells with p24-siRNA enclosed in lipids, which are ideal for getting small molecules into cells. "Using lipids probably isn't practical for *in vivo* use," Dr. Lieberman commented, "but it's a good way to experiment with siRNA *in vitro* to see if it works." Upon successfully transfecting the cells with p24-siRNA, the cells were infected with HIV 24 hours later. Two days after infection, flow cytometry indicated that the p24-siRNA-tranfected cells showed a greater than fourfold decrease in viral protein, compared with controls. Five days after infection, flow cytometry determined that 62% of the control CD4+ cells were expressing p24, compared to 14.4% of cells transfected with p24-siRNA.

When HIV expresses its genes within cells, it produces different kinds of messages. The long message—the full-length HIV transcript—serves as both the mRNA for the gag-pol genes and the genomic RNA of progeny virus. The shorter messages include nonstructural regulatory genes, including tat, rev, and nef, which are expressed from the provirus before integration into the host genome. "By silencing p24, we observed a reduction, not only in the long message, but also the shorter messages," Dr. Lieberman pointed out. "What we think is going on is that we may be able to target the viral genome, as the virus enters the cell after binding to a receptor and fusing with the host-cell membrane. This single-stranded RNA, we believe, is one target of the gag siRNA. If it doesn't get targeted and the viral RNA is reverse transcribed and the viral genome is

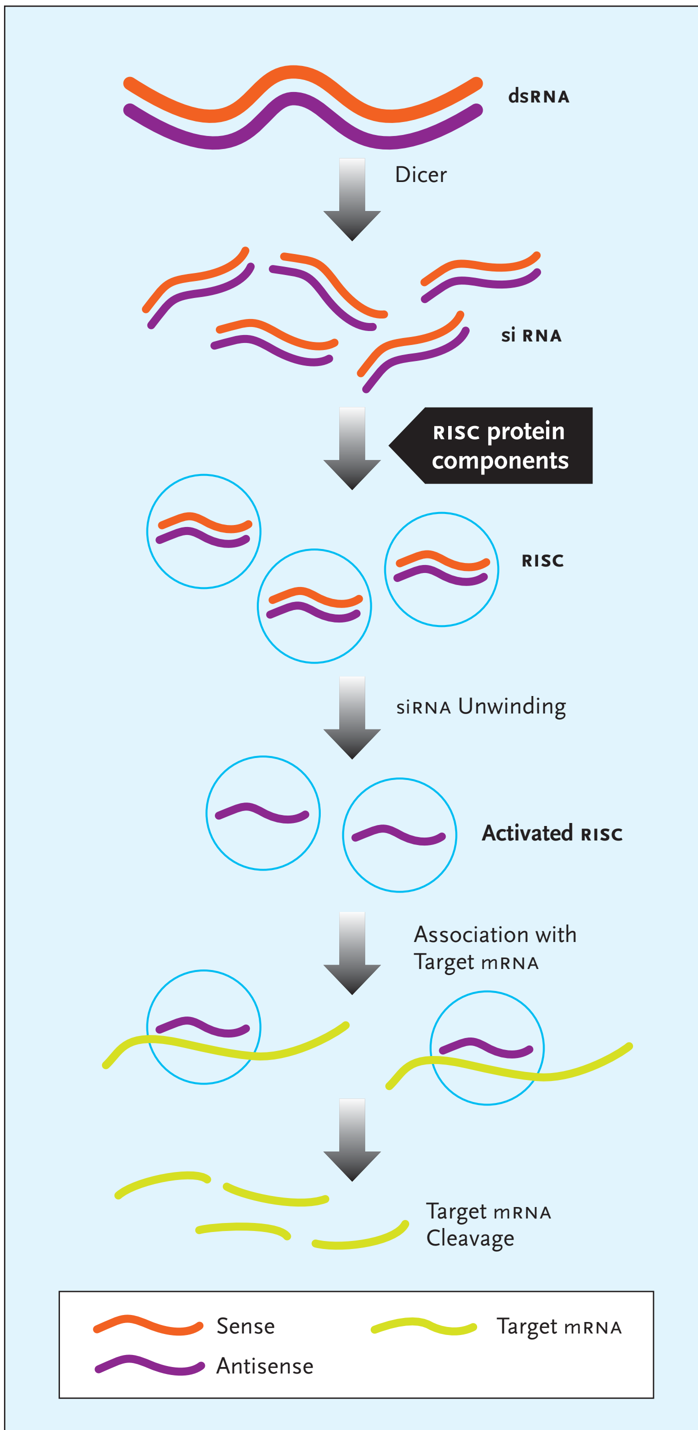


Figure 1. **RNA Interference: Step by Step**

Short interfering segments of double-stranded RNA (siRNA) are produced when the enzyme Dicer—a member of the Rnase III family of double-stranded RNA (dsRNA)-specific ribonucleases—cleaves dsRNA to form degraded siRNAs containing approximately 19 base pairs each and approximately 21 to 23 nucleotides in length. From there, siRNAs are assembled into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs). The siRNA strands are then unwound to form activated RISC. The siRNA strands subsequently guide the RISCs to messenger RNA (mRNA)—cleavage of mRNA takes place near the middle of the region bound by the siRNA strand—ultimately silencing the intended actions of the gene.

Souce: Ambion, Inc.

integrated into the host DNA, then we have a second chance to target the virus, when its genes are expressed from the genomic DNA—to target the different messages before new virions are produced."

Silencing HIV in T-cells and Macrophages

SILENCING HIV IN LABORATORY-DERIVED CELL LINES IS ONE THING. Silencing HIV in primary T-cells and macrophages is another situation entirely. "We've been moving step by step to determine if RNA interference can be used as a therapy or possibly a microbicide to prevent HIV transmission," Dr. Lieberman explained. "This required moving beyond our initial experiments using artificial cell lines and into experiments to determine if we could silence HIV infection in primary T-cells and macrophages."

In the first experiment conducted by Dr. Lieberman's group, a line of primary T-cells were transfected with siRNA against green fluorescent protein (GFP) (Novina, 2003). These cells were then infected with a strain of HIV in which the nef gene was removed and replaced with GFP. In other words, the lower the GFP expression observed in these T-cells, the greater the impact of siRNA on the silencing of HIV in these T-cells.

Two days after siRNA transfection and HIV infection, reductions in p24 and GFP expression were detectable. Within five days, there was a three to fourfold reduction in these HIV proteins. By the ninth day, HIV proteins were once again being expressed on a par with control levels. "In primary T-cells, the HIV silencing lasts approximately five days," Dr. Lieberman said. "We were only able to silence HIV expression by a third in these T-cells, which is less that what we saw using the artificial cell lines. The problem is, primary T-cells are difficult to transfect."

In a more recent experiment, published in the *Journal of Virology*, a group that included Dr. Lieberman and led by Dr. Premlata Shankar set out to determine if more sustained siRNA-mediated silencing of HIV is possible in macrophages, which, like T-cells, constitute an important reservoir of HIV *in vivo* (Song, 2003). They used siRNAs that targeted CCR5—the major HIV coreceptor on macrophages—and Gag to squelch p24 production, both individually and together. Two days after transfection, the macrophages were infected with M-tropic virus. Both CCR5 and p24 siRNAs effectively reduced HIV expression for the entire 14-day period of observation. What's more, when siRNAs targeting CCR4 and p24 production were combined, infection was virtually abolished.

Dr. Lieberman's group also wanted to determine if exogenously introduced siRNA remains stable in macrophages. To do this, they tested the kinetics of siRNA-mediated viral inhibition by initiating HIV infection at various times, between two to 15 days after transfection with siRNA. The silencing of HIV mediated by p24 siRNA progressively decreased and was lost by the seventh day post-transfection. Conversely, silencing of CCR5 expression was sustained, even when transfection preceded infection by 15 days, indicating that the continued presence of target RNA may be needed for persistence of siRNA.

What about cells that are first infected with HIV and then transfected with siRNA, a situation that more accurately applies to the potential utility of RNAi as an HIV therapeutic? According to the *Journal of Virology* paper, long-term suppression of HIV replication—in macrophages already infected with HIV—can be achieved with p24-siRNA.

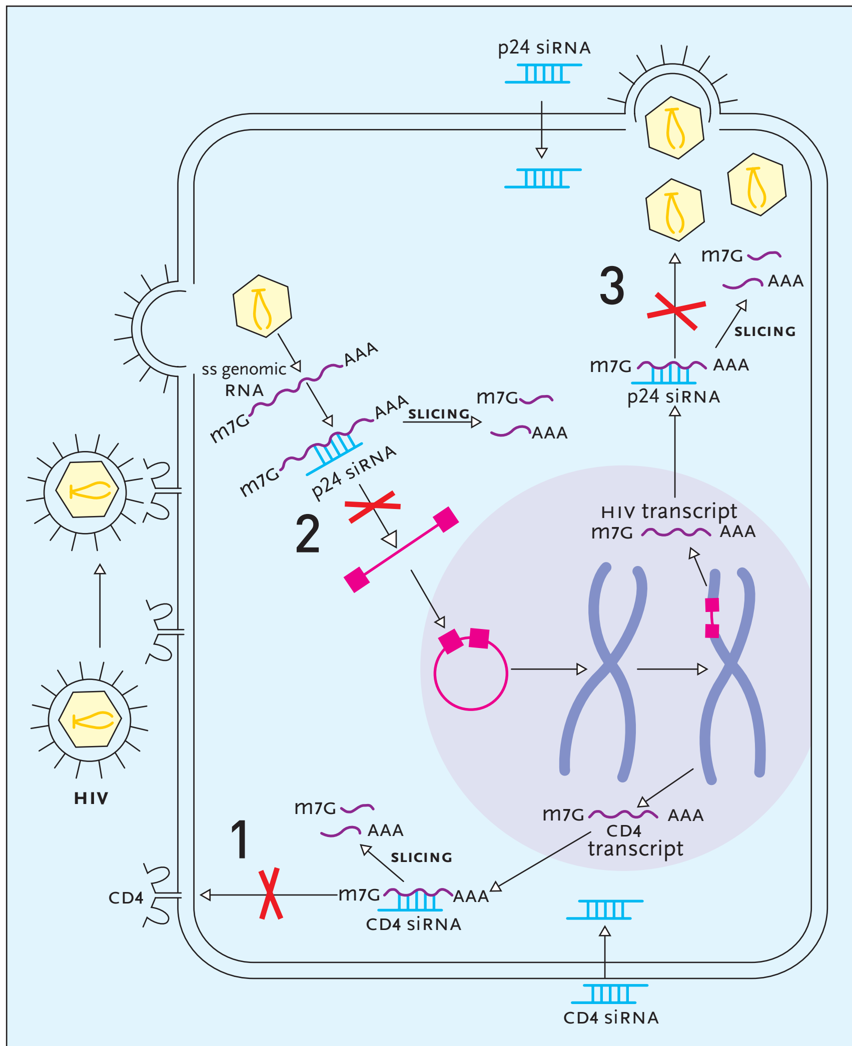


Figure 2. **RNA Interference: Therapeutic Potential in HIV Infection**

A model depicting the various potential pathways of RNA interference to inhibit HIV infection and production in CD4+ cells. siRNA directed to the viral receptor mRNA inhibits virus entry into cells (Step 1). Silencing of preintegrated HIV may occur by p24 siRNA targeting the RISC complex directly to the HIV genome to prevent integration (Step 2). In addition, HIV progeny virus production may be inhibited by silencing full-length HIV gene expression (mRNA or genomic RNA) expressed from the integrated virus (Step 3).

Source: Novina CD, Murray MF, Dykxhoorn DM, et al. **siRNA-directed inhibition of HIV-1 infection.** *Nature Med* 8(7):681-6, 2002. Reprinted with permission of *Nature Medicine* and the Nature Publishing Group.

RNAi in Autoimmune Hepatitis: First Evidence of a Potential Therapy

BECAUSE THERE ISN'T AN ADEQUATE SMALL-ANIMAL MODEL FOR HIV infection, Dr. Lieberman and her colleagues have broadened their focus to include diseases that can be studied and targeted using RNAi in rodents. "We set out to target the gene Fas, which encodes the Fas receptor, to see if it protects mice from liver failure and fibrosis in two mouse models of autoimmune hepatitis," Dr. Lieberman explained.

Many liver diseases are characterized by apoptosis, which is mediated by the protein Fas. Whether it is autoimmune hepatitis, viral hepatitis, or transplant-rejection hepatitis, significant damage to the liver is primarily caused by the infiltration of the liver by activated lymphocytes, which stimulate the Fas receptor and ultimately trigger massive apoptosis in hepatocytes. Engaging Fas also provokes hepatic inflammation by inducing expression of hepatic chemokines that recruit and activate immune cells, which further perpetuates the cycle of liver damage. "It's not the hepatitis C virus or the hepatitis B virus causing liver disease," Dr. Lieberman said in offering an example. "It's the immune response that does the damage by tickling the death receptor Fas on the surface of liver cells, which we can target using siRNA."

By bolus injection, Dr. Lieberman's group administered Fas siRNA into the tail veins of several mice (Song, 2003a). Using fluorescent labeling (Cy5), they first wanted to determine whether or not the siRNA was taken up into the hepatocytes. Sure enough, 24 hours after the last of three 50 mg Fas siRNA injections, 88% of the hepatocytes were found to be Cy5-positive, meaning that uptake was successful. "This goes to show that we had efficient delivery and uptake by hepatocytes, which is what we wanted to see," Dr. Lieberman commented.

Because Cy5-labeled siRNA does not induce gene silencing, Dr. Lieberman's team conducted additional experiments using an RNase protection assay (RPA) to measure Fas mRNA and also measured protein expression in the hepatocytes of mice receiving Fas siRNA through their tail veins. Accordingly, treatment with Fas siRNA reduced Fas mRNA expression eight to tenfold, compared to control injections, 24 hours after injection. Fas mRNA was silenced out to ten days post-injection, with Fas mRNA expression reappearing around the 14th day post-injection. By the 21st day post-injection, Fas mRNA expression was back to normal.

Dr. Lieberman pointed out that the injections of Fas siRNA were highly specific, meaning that they only targeted Fas. They did not affect the expression of other Fas-related genes. "We observed very specific, long-lasting activity with these injections," she said. "This is an ideal situation."

To determine if this silencing of Fas actually provides a protective benefit in terms of preventing fulminant hepatitis, mice were once again injected with Fas mRNA (or saline or GFP siRNA, which served as the controls) and challenged, 24 hours later, with high-dose injections of concanavalin A (ConA), a plant lectin that enters the liver and summons an influx of lymphocytes that trigger apoptosis (see Figure 3). Approximately 20 hours after exposure to ConA, the mice were sacrificed so that analyses of liver pathology and serum transaminases could be performed.

All saline-treated and GFP siRNA-treated mice had extensive liver damage caused by apoptosis, indicated by the significant hepatocytes necrosis, bridging and inflammatory infiltrates surrounding both the portal and central veins, and extensive cytoplasmic swelling among the surviving hepatocytes. In contrast, Fas siRNA prevented hepatocytes necrosis and abrograted inflammatory infiltration in the treated mice, although some mild hepatic swelling was observed. Serum AST and serum ALT levels were significant elevated in the control mice, compared to the Fas siRNA-treated mice.

Dr. Lieberman's group also wanted to look at the therapeutic effect of Fas siRNA under circumstances that more closely mirror chronic hepatitis. This involved delaying Fas siRNA treatment until 24 hours after the second of six weekly injections of reduced-dose ConA. Mice were killed five weeks after receiving the first of two Fas siRNA injections and one week after receiving the last ConA injection (week seven of the

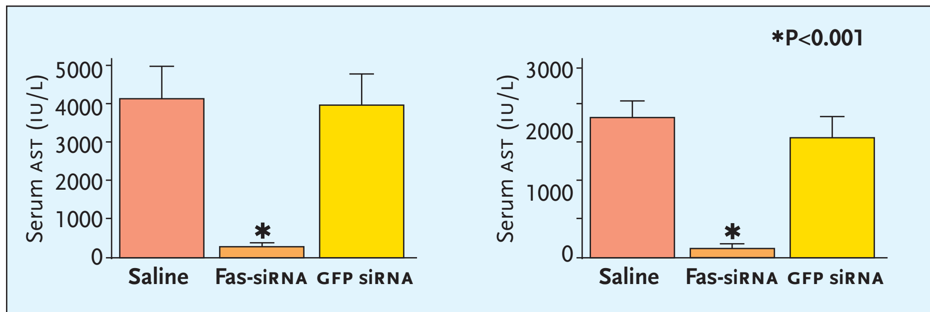


Figure 3. **RNA Interference: Reduced Hepatocyte Death in Mice with Induced Hepatitis**

Mice were injected with Fas mRNA (or saline or GFP siRNA, which served as the controls) and challenged, 24 hours later, with high-dose injections of concanavalin A (ConA), a plant lectin that enters the liver and summons an influx of lymphocytes that trigger apoptosis. Serum AST and serum ALT levels were significant elevated in the control mice, compared to mice treated with fas siRNA, designed to protect the Fas receptor responsible for triggering hepatic inflammation and massive apoptosis in hepatocytes.

Source: Song E, Snag-Kyung L, Wang J, et al. **RNA interference targeting Fas protects mice from fulminant hepatitis.** *Nature Med* 9(3):347-51, 2003a. Reprinted with permission of *Nature Medicine* and the Nature Publishing Group.

study). All control mice developed bridging fibrosis in the liver parenchyma, whereas no fibrosis or necrosis was seen in the livers of mice treated with Fas siRNA. Chemical indicators of active liver disease—including hepatic hydroxyproline and serum procallagen type III—were significantly elevated in the control mice, compared to Fas siRNA-treated mice. "The liver pathologies and biochemical markers for the treated mice were virtually normal," Dr. Lieberman commented. "What's more, we didn't find any evidence of toxicity, splenomegaly, or other organ damage in the mice receiving repeated injections of Fas siRNA."

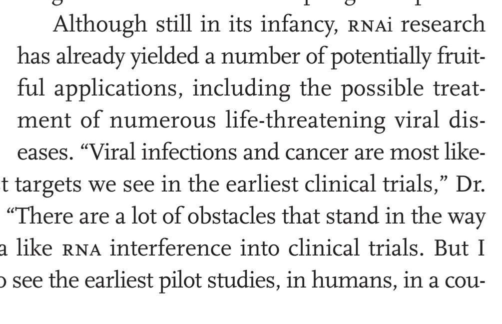
The final experiment discussed by Dr. Lieberman focused on the lifesaving benefits of Fas siRNA in a more aggressive hepatitis model: mice receiving an intraperitoneal injection of a Fas-specific antibody. All of the control mice injected with the antibody were dead within three days, with most of the mice dying within 24 hours after receiving the antibody. Some of the designed Fas siRNAs did not silence Fas expression effectively and all the mice receiving those siRNAs also died. "However, 80% of the mice with at least 80% silencing of Fas survived," Dr. Lieberman said. "This is basically life and death protection, which I think suggests that this is a really promising technology."

Conclusion

IN SUMMARIZING HER GROUP'S RESEARCH AND PROVIDING A GLIMPSE AT what these data may hold for future research Dr. Lieberman pointed out the obvious: that man is no mouse. "Can we go from a mouse weighing 20 grams to a person," she questioned? "The problem is, we can't really scale up the injections used in mice. You can't inject a liter of fluid into a human in a few seconds like we did with the mice, and I doubt that we can transduce the human liver very efficiently using this method. It is conceivable that we might be able to achieve this with regional catheterization of the portal vein. But the truth is that I don't think it's going to happen like this. I think it's going to be extremely difficult, maybe even impossible, to get these sort of infusions to work in monkeys or people."

The mode of administering siRNA into humans is a lingering challenge. One approach currently being explored is the expression of siRNA from a vector, similar to what is being done in gene therapy, using a lentivirus or a retrovirus. "This approach can work," Dr. Lieberman suggested. "We've made lentiviruses that can express siRNAs that target HIV long terminal repeat, rev, and gag. They all work. But this approach is associated with a number of problems. One obstacle is achieving efficient transduction of cells. Another problem is getting the cells that are efficiently transduced to express the gene of interest for long periods of time." Another, perhaps more significant problem is the risk of malignancy. Lentiviruses insert genetic material into the most active part of chromosomes, where oncogenes exist. "I, personally, don't favor gene therapy," Dr. Lieberman confessed. "But it's an approach that we're continuing to look at and are attempting to improve."

Although still in its infancy, RNAi research has already yielded a number of potentially fruitful applications, including the possible treatment of numerous life-threatening viral diseases. "Viral infections and cancer are most likely going to be the first targets we see in the earliest clinical trials," Dr. Lieberman surmised. "There are a lot of obstacles that stand in the way of translating an idea like RNA interference into clinical trials. But I wouldn't be shocked to see the earliest pilot studies, in humans, in a couple of years." *prn*



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