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Determined stance: Robert Place (left) and Long-Cheng Li (right) vow to continue their controversial work on RNA activation.

Hitting the on switch

Researchers in San Francisco have findings that suggest a whole new side to RNA interference. **Erika Check** reports on their attempts to make a revolutionary field more revolutionary still.

"That looks perfect," murmurs Robert Place as he watches a smooth line trace across his monitor. "It never works this well."

Place, a molecular biologist at the Veterans Affairs Medical Center and University of California, San Francisco, is using a spectrophotometer to measure the purity of a series of samples. Every sample contains a tiny drop of micro RNA (miRNA), a type of genetic regulator that dampens gene expression — or so the story goes. The experiment Place is absorbed in is the last he must complete before he submits a publication that could upend that story. He and his colleague, Long-Cheng Li of the University of California, San Francisco, think they have found some miRNAs that boost, rather than silence, gene expression in cells.

Their work could shake the foundations of one of the hottest topics in biology — RNA interference — which studies how short pieces of RNA regulate the expression of genes. Place knows that his experiment will draw intense scrutiny from other researchers, and therefore it has to go perfectly. And as far as he can see, it has. His spectrophotometer displays a series of flawless curves, free of impurities; it looks as if he and his colleagues are finally seeing the pay-off after a three-year saga of frustration and exhilaration.

Since about the turn of this century, scientists have realized that 50 years of focus on DNA had blinded them to the wide range of biological roles held by its chemical cousin, RNA. The old view was that DNA contained life's instructions, proteins carried them out, and RNA served as little more than a go-between. It's now become clear that RNA has vast potential for controlling how cells interpret the instructions embedded in the genome.

> **"It was so easy to observe activation that I didn't understand how people could have ignored it."**
> — Long-Cheng Li

The RNA revolution began in 1998 with the discovery honoured by last year's Nobel Prize in Physiology or Medicine that small strands of RNA stuck together in pairs, like the strands that make up DNA, could turn off specific genes in roundworms[1]. RNA interference was born.

In 2001, scientists discovered that the process works in mammals, too[2]. They found that interference could be triggered by 'short interfering RNAs' (siRNAs) or 'short hairpin RNAs' (shRNAs). Both work by using a pair of scissors, actually a complex of proteins known as RISC, to cut apart the longer messenger RNAs (mRNAs) that take information from the genes to the cell's protein-making machinery. The small RNAs target mRNAs because their sequences match, and by destroying the messenger, RNA interference stops genes from making proteins. Both siRNAs and shRNAs are being tested in clinical trials against conditions in which an overactive gene needs to be shut down[3].

Scientists then discovered miRNAs, which also trigger the interference pathway, but unlike siRNAs and shRNAs are naturally encoded by cells' DNA. So far, more than 500 miRNAs have been identified in the human genome[4]. They can act like siRNAs, using the same protein complexes that slice through messenger RNA. But for the most part, miRNAs are content to muzzle the message or mark it for degradation, rather than chop it up (see graphic).

All these techniques were still quite new back in 2004, when Li began to investigate RNA at the San Francisco Veterans Affairs Medical Center in the laboratory of Rajvir Dahiya, a urologist. Li, also a urologist, was studying epigenetics — stable modifications to the genome that change how it is read without altering its sequence.

He was particularly interested in DNA methylation, the addition of chemical tags called methyl groups to regions of DNA. Methyl tags often silence nearby genes, which can be disastrous for genes that usually suppress tumours. So Li was trying to find ways to reverse this process. He decided to try an RNA-based technique to control methylation that hadn't yet been used in animals, as far as he knew, although it had been demonstrated in plants a decade earlier[5].

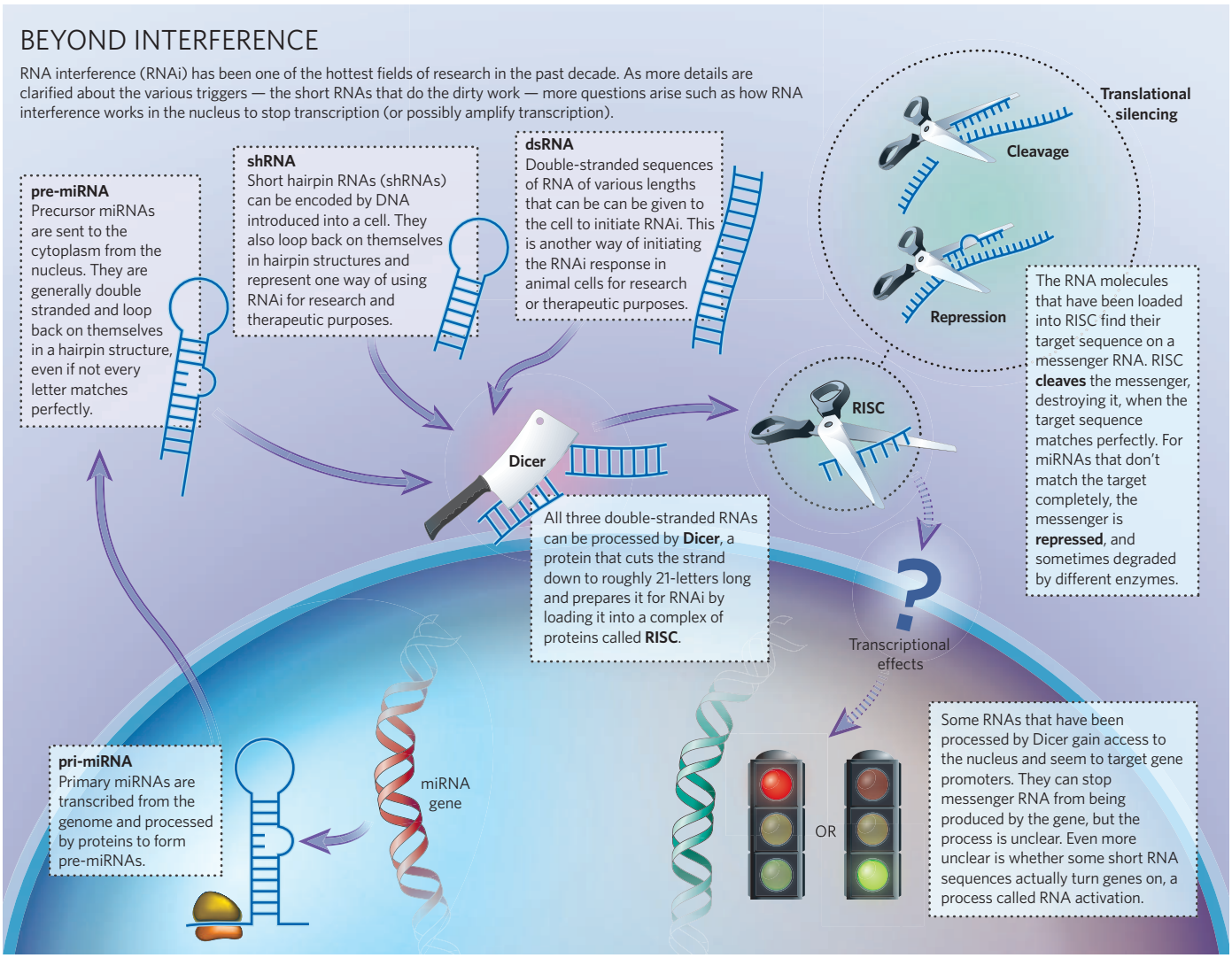
Unexpected boost

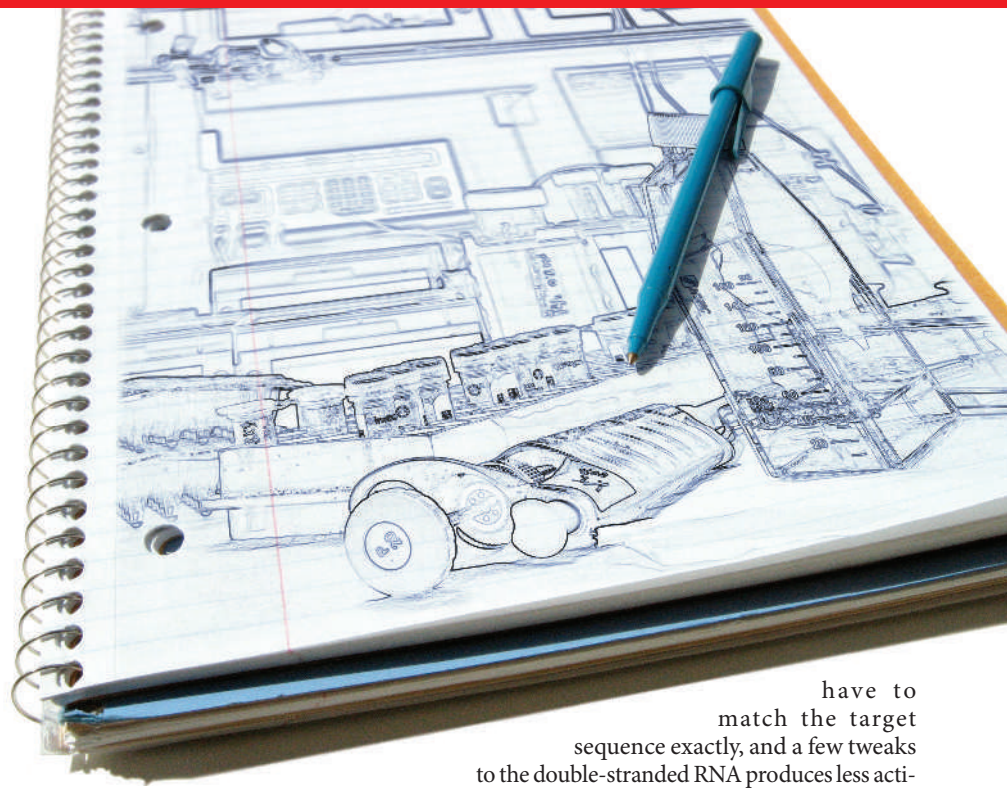
Li purchased two pieces of double-stranded RNA that were complementary to a DNA control sequence — known as a promoter — upstream of the gene that encodes the tumour-suppressor protein E-cadherin. Then, his colleague Hong Zhao added the double-stranded RNA to two lines of prostate cancer cells and measured how it affected E-cadherin expression. Three days later, Zhao noticed something shocking: the experiment had boosted levels of the E-cadherin protein by 4- to 14-fold. "I couldn't believe it," Li says.

The work defied everything that scientists had ever reported about small, double-stranded RNAs. They were only supposed to dampen gene expression. Li seemed to have stumbled on a phenomenon that could rewrite the textbook understanding of RNA interference and might offer new therapeutic potential.

Li re-ran the experiment many times, each time with the same result. He found that two other cancer-related genes, *VEGF* and *p21*, could also be activated by double-stranded RNAs. The evidence seemed solid. At least in the prostate cancer cells he was working with, short, double-stranded RNAs activated genes. "It was so easy to observe — I didn't understand how people could have ignored it for such a long time," Li says.

But apparently they had. So Li knew he was going to have to put together a watertight case to convince other scientists. He started to realize how difficult that would be when he first submitted his work for publication to *Science* in August 2004. It was promptly

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rejected. He then submitted his paper to *Nature* that December; when it was rejected, he resubmitted it with new data in April 2005. He presented his findings at the annual conference of the American Association for Cancer Research in Anaheim, California, in May 2005, and the reception wasn't warm. "I got a lot of sceptical questions," Li says. Then, after an extensive delay, *Nature* again rejected Li's paper in December, 2005. Without evidence for a mechanism, he says he was told the results weren't convincing enough.

Unsure how to proceed, Li got some unexpected help in the form of Place, who joined the lab in October 2005. Fresh from earning his PhD, Place was excited by the novelty of Li's findings. But he could tell that the lab had a lot of nitty-gritty molecular biology work to do. Place helped plan experiments to help convince the sceptics. For instance, he helped Li and his technician Deepa Pookot to perfect assays, called immunoblots, that detect proteins so that they could measure how RNA activation boosts levels of E-cadherin and other proteins.

Filling in the details

Place and Li also started to think about the mechanism behind RNA-directed gene activation, a major stumbling block for the editors and reviewers who had seen the paper. If it worked in the same way as gene silencing, the sequence of the trigger RNA should matter, and the pair found that it did. Changing five letters at one end of the 21-letter sequences rendered them inactive.

The pair also found that activation used some of the same key proteins that are involved in silencing, such as Dicer, which cuts up strands of RNA so that they can be used by RISC to target mRNA. They began to experiment with activation, trying to find out what worked best. "We started to modify the RNA duplexes to get optimal activation, tweaking their chemical structures," Place recalls. "There came a point when we were working in synchronization, and we really started to click."

> **"Last time I talked about activation at a meeting, a couple of leaders in the field jumped all over me and said it wasn't true."**
> — David Corey

Li resubmitted the work to *Science* with Place's additional molecular biology results, but it was again rejected. The letter he received said that because the work "would represent a substantial paradigm shift", the evidence just wasn't strong enough. Again, editors required demonstration of a mechanism.

So, in June 2006, Li talked to leaders of the RNA-regulation field at a meeting in Cold Spring Harbor Laboratory, New York. Li recalls asking David Bartel of the Whitehead Institute for Biomedical Research in Cambridge, Massachusetts, if he thought RNA activation — not just inhibition — was possible. Bartel said he didn't think so, Li says. Bartel says he doesn't remember the conversation, "I was probably just trying to find out how solid the evidence for activation really was," he explains. Li was after all a junior researcher in an unrelated field, and he hadn't been able to publish his findings.

Exasperated, Li considered sending his findings to an online journal with less stringent criteria than *Nature* or *Science*. But Dahiya thought that the work would get buried in obscurity, and convinced Li to try the *Proceedings of the National Academy of Sciences* instead. In August 2006, the lab submitted its work there — and finally got its breakthrough. The journal reviewed it in weeks and published the work online in November[6]. Two months later, vindication: another lab, led by biochemist David Corey of the University of Texas Southwestern Medical Centre in Dallas, published a paper essentially duplicating the results in *Nature Chemical Biology*[7]. "It was really good for us," Place says. "Before that, it seemed like everyone just thought we were crazy."

Back at the Veterans Affairs Medical Center, Place and Li were now working closely together, although Li was preparing to move to his own lab at the University of California, San Francisco. They had already devised a series of experiments that they hoped would bolster their activation hypothesis. Although some changes to the sequence can render RNA useless, others fine-tune the activation. The trigger doesn't have to match the target sequence exactly, and a few tweaks to the double-stranded RNA produces less activation. And that's exactly how miRNA works. So the pair started hunting for a miRNA that might activate E-cadherin. It wouldn't prove that activation was a natural phenomenon, but it would strengthen the case, because miRNAs are encoded in the genome.

Place and Li used a bioinformatics tool to search for miRNA in the human genome that had sequences roughly complementary to the E-cadherin promoter sequence. They found a handful of candidates and transferred them into the prostate cancer cell lines. The experiment was so simple they half-expected it wouldn't work — but it did. The pair found one miRNA, miR-373, that boosted E-cadherin expression. "When that worked, we were totally pumped, because it was a potential example of natural function," Place says.

On the right track

But it still wasn't proof. So Place designed an miRNA precursor that, according to the model of miRNA biogenesis, should work as well as the miRNA itself. It did. Place also found that if he knocked out Dicer, then miR-373 stopped working, and that Dicer could also activate another protein with a promoter sequence similar to E-cadherin's. All these experiments supported the idea that miRNA could use the interference pathway to activate genes. It was a strong hint that Li and Place were on the right track. On 6 August, they submitted the work to *Nature Chemical Biology*.

The question now is how this work will be received. Many are still sceptical about activation, as David Corey can attest. "Last time I talked about this at a meeting, a couple of leaders in the field jumped all over me and told me it wasn't true," Corey says. "We've received grant and manuscript reviews that seem to express irritation more than anything else. I

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In this together: Long-Cheng Li (left) is briefly reunited with the team from the Veterans Affairs Medical Center. Left to right, Li, Emily Noonan, Robert Place, Deepa Pookot and Rajvir Dahiya.

just wish they would look closely at our data."

Indeed, it sometimes seems as though the idea of activation is struggling because it contradicts the interference dogma. The RNA interference field is quite young, but already seems to have acquired a certain amount of inflexibility. On scanning through archives of biology message forums, Place has found that other scientists — often graduate students — have also seen evidence of RNA activation. But they have been encouraged to discount it.

Phillip Sharp, a Nobel-prize-winning biologist whose lab at the Massachusetts Institute of Technology in Cambridge pioneered much of the work on RNA interference, admits that RNA-mediated activation might be possible, but says that "the results I have seen do not prove this". Echoing the demands of manuscipt editors, he says, "I think further papers on the subject must address the mechanism if they are to be published in a high-profile journal."

"Further papers on the subject must address the mechanism if they are to be published in a high-profile journal."
— Phillip Sharp

A new pathway

As the field has moved apace, such requests are warranted, and providing proof of a mechanism is Li and Place's main challenge. Some have suggested that activation is simply inhibition in disguise. It could be the accidental result of silencing an upstream repressor or of blocking another silencing RNA. Although they can't rule out these possibilities, from their experiments, they say that these mechanisms look unlikely. For instance, they can elicit activation predictably at specific genes by targeting their promoters.

There are intriguing differences between the known silencing pathways and the observed activation phenomenon. Silencing is triggered within hours and ceases in about seven days, whereas activation takes days to appear but can last for weeks. The different kinetics suggest that some mystery process is involved, Place says. "People say this could be RNA interference with another name, but it's so blatantly different."

Major questions also remain about how RNA regulation might act at gene-promoter regions. In the classic interference pathway, the RISC complex guides siRNAs or miRNAs to a target mRNA in the cell's cytoplasm. But to regulate a promoter, a small RNA would have to sneak into the cell nucleus, where DNA is transcribed. There is mounting evidence that this happens in silencing pathways, but again the mechanism is unclear.

In 2004, two groups published papers that suggested that siRNAs that target gene-promoter regions can silence genes if they are delivered into cell nuclei. Although one group has since retracted its paper, the other group, who published in *Science*[8], showed that the inhibition was accompanied by epigenetic marks associated with silencing. Kevin Morris, of the Scripps Research Institute in La Jolla, California, an author of that paper, has continued to study how this occurs, and sympathizes with Li and Place's position. "I was there in 2004, when 50% of the people love your work and the other 50% think you're full of it," Morris says. "It's a frustrating place to be."

Frustrating, indeed. As the deadline for this news feature approached, Place received word that the group's manuscript on miRNA activation had been rejected. The reason given: without proof of a mechanism, the evidence isn't substantial enough. Li is still adamant that the field will come around. "We have no doubt that RNA activation is an endogenous mechanism," he says. And Place seemed unsurprised. "We knew the mechanism would be the sticking point. That's the hardest part to prove." But, he predicted, if the group can just get someone to just take a look at its data, the strength of its evidence will prevail. "If we can get it into review, we'll be okay," Place says.

When RNA interference first hit the scientific radar, it was a slow climb from something written off by many as artefact to a revolutionary paradigm. That same uphill battle confronts Place, Li and their collaborators as they try to rewrite, or at least refine, the revolution. Call it what you will — stubbornness, confidence or optimism — this group just isn't going to give up. ■

Erika Check writes for *Nature* from San Francisco.

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