# RNA interference: unraveling a mystery

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**Andrew Fire and Craig Mello have won the Nobel Prize in Medicine or Physiology for their discovery of RNA interference. Mary K. Montgomery, then a postdoc in the Fire laboratory, participated in some of the key experiments.**

This year's Nobel Prize in Medicine or Physiology was awarded to Andrew Fire and Craig Mello for their work on RNA interference (RNAi). I was privileged to be a postdoc working in Andy's lab at the Carnegie Institution of Washington's Embryology Department (which, despite its name, is located in Baltimore, Maryland) from 1994 to 1998 and to contribute the results of a few experiments included in the 1998 *Nature* paper cited by the Nobel committee[1]. But all such work builds upon previous studies, and the brilliant insight that seems to come in a flash is often preceded by much rumination, speculation, testing, false starts, false leads and revision of ideas; and so the story begins long before my arrival in Andy's laboratory.

In 1991, Andy published a paper describing successful attempts to disrupt *Caenorhabditis elegans* gene function using transgenes designed to generate antisense RNAs targeting the gene of interest[2]. The transgenic approach was similar to that pioneered by Izant and Weintraub for mammalian cell lines several years previously[3]. Notably, however, Andy and his coauthors reported that injection of sense constructs containing sequences of the gene under study gave rise to the loss-of-function phenotype at a frequency close to that of the antisense constructs. At the time, however, this result was thought to be due to production of antisense RNA from indiscriminate transcription off the transgenic extrachromosomal arrays that carried multiple copies of the sense construct. The prevailing wisdom at the time was that loss-of-function phenotypes could be induced by introducing antisense RNAs complementary to the messenger RNA product(s) of the targeted gene. The gene's product (mRNA) was thought to base-pair with the introduced antisense RNA, preventing production of the encoded protein through one or more mechanisms such as blocking synthesis, processing, export or translation of the targeted mRNA, or recruiting double-stranded (ds) RNA nucleases that would degrade the antisense-mRNA duplex. The observation that sense RNAs could produce a similar effect was intriguing but confined to just one of two genes used in the study, and the results did not seem to rule out an interpretation that supported an 'antisense-inhibition' model.

A few years later, though, Su Guo and Ken Kemphues[4] reported a similar finding when they used an antisense strategy to confirm the molecular identity of the maternally expressed *par-1* gene. In this study, Guo and Kemphues injected *in vitro*–synthesized antisense RNAs directly into the worm's syncytial gonad and obtained phenotypes resembling *par-1* maternal loss-of-function mutants (that is, phenocopies of the mutants). Surprisingly, in a subset of their experiments, injection of the sense RNA control was equally effective at inducing the *par-1* phenocopy. Guo and Kemphues chose not to speculate on a possible mechanism that would explain the sense results. Although several interpretations were plausible, the results argued against a simple antisense-inhibition model.

Other *C. elegans* researchers, particularly those working on maternally expressed genes, quickly adopted the antisense-injection method described in the Guo and Kemphues paper as a useful reverse-genetic technique, despite a lack of understanding of the mechanism underlying the method. Maternal genes were thought to be particularly amenable to this technique, as a single injection into the gonad would at least dramatically reduce the targeted maternal mRNA, resulting in the production of up to a hundred or more affected embryos. The typical *C. elegans* adult animal is a self-fertilizing hermaphrodite that contains two 'U'-shaped gonads, so just two injections into an adult were thought to be sufficient to produce an entire brood of affected embryos.

Craig Mello was a young investigator fresh out of Jim Priess's laboratory, setting up his own shop at UMass Medical Center, in 1994. By 1996, Craig and his student Sam Driver began reporting on several puzzling aspects of the reverse-genetic methodology they and others were using to examine gene function. Like Guo and Kemphues, they noted that injection of sense RNAs could often mimic the antisense phenotype. Moreover, injection into just one gonad arm could produce phenocopies in the entire brood, as if the interfering RNA or its effects were being distributed beyond the site of injection. (Craig could not believe that Sam, as a novice injector, could be hitting both gonads so precisely, and together they systematically showed that the RNAi effect must be transmitted to other tissues. The mechanism of this 'systemic spreading' of RNAi in worms remains an active area of investigation[5].) Even more puzzling was the observation that, in some cases, the loss-of-function effect was heritable, lasting for several generations. This was hypothesized to result from the formation of extrachromosomal arrays from injected material; initially, it was thought that the injected RNA was being reverse-transcribed in the worm germ line. (The heritable aspect of RNAi is uncommon and still not fully understood; see further discussion below.)

Again, all the evidence indicated that a simple antisense-inhibition model was not responsible for the loss-of-function effects, and thus Craig coined the term 'RNA interference' to distinguish the phenomenon he and coworkers were describing. As Craig

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explained in an informal workshop at the 1997 Worm Meeting in Madison, Wisconsin, "We can't call it 'antisense' when 'sense' works as well." Craig's laboratory also identified several other crucial aspects of the interfering RNA: intronic and promoter sequences did not work, and neither single-stranded (ss) DNA nor dsDNA seemed to produce much of an effect. The mystery was in full swing.

Andy's main research interests during his years at the Carnegie revolved around understanding the role of gene regulation in specifying cell fate, and by the mid-1990s, a few of us in the lab were specifically working on regulation of maternal RNAs. I spent the first three years of my postdoc in Andy's lab working with him on improved methods for transgene expression in the germ line, a notoriously recalcitrant tissue. We hoped to develop a transgenic assay for testing the potential regulatory roles of 3′ untranslated regions in maternal-RNA translation and stability.

Earlier in their careers, Andy and Craig had each contributed toward improving methods for DNA transformation in worms[6]. Andy's and Craig's paths began crossing again in 1997 owing to a mutual interest in the role of a gene called *pie-1* (ref. 7). Inevitably, discussions broadened to include both Andy's and Craig's experimental results with antisense and RNAi and what it all might mean. After hours of phone conversations, the idea emerged that what the 'sense' and 'antisense' RNA preparations might have in common is that each might be contaminated with RNAs synthesized off the opposite strand, owing to the less-than-perfect fidelity of the viral RNA polymerases used in *in vitro* transcription reactions. In other words, each prep could be contaminated with small amounts of dsRNA. Could dsRNA, rather than antisense or ssRNA, be the effector molecule responsible for RNAi effects?

When it was presented during lab meeting, the idea was greeted with a healthy skepticism, because a mechanism by which low levels of dsRNA could cause interference was not immediately obvious. And weren't cells supposed to rapidly degrade dsRNA? Nonetheless, the idea was pursued over the next few weeks. Andy, along with SiQun Xu, our talented lab technician, went about testing the relative effectiveness of highly gel-purified preparations of ssRNAs, compared with preparations where the two strands were deliberately annealed to form dsRNA. The results were unequivocal. The dsRNA was vastly more effective at causing specific loss of function of the targeted gene (*unc-22*, the same gene used for the 1991 studies) than either of the purified single-stranded preps. Moreover, the dsRNA was amazingly potent: Andy calculated that, in some experiments, the presence of only a very few dsRNA molecules per cell was capable of causing interference.

One aspect of the mystery seemed solved; but as is typical in science, ever more sleep-depriving questions emerged. Immediately, a few of us became intensely interested in gaining some insight into the mechanism(s) by which dsRNA could be causing interference. How to explain its remarkable potency? Interference on a transcriptional level seemed to be an obvious answer, although other models were possible. A reasonable next experiment was to see what happened to the mRNA associated with a targeted gene after treatment with dsRNA. I performed a series of experiments targeting *mex-3*, which is abundantly transcribed in the adult maternal germline; dsRNA (or purified sense or antisense RNA) was injected into young adults, and then the animals and their embryos were processed for *in situ* hybridization. Andy and I half-expected to see the targeted mRNA sequestered in the mysterious germline P-granules. Instead, we found that the abundant endogenous *mex-3* mRNA decreased below the level of detection within a few hours of dsRNA exposure, but not after delivery of either purified sense or purified antisense RNA. These results, along with those demonstrating that only sequences from the coding portions of a gene were effective at causing interference, pointed to a post-transcriptional mechanism. Still, the *mex-3* experiments couldn't definitively rule out the possibility that we were observing shutdown of gene transcription and normal turnover of the remaining mRNA. We addressed this question in greater detail in follow-up experiments, which strongly supported the view that RNAi in worms works primarily at a post-transcriptional level[8].

Andy also designed several clever hybrid constructs to test whether dsRNA could potentiate an antisense effect; all the resulting additional evidence supported the view that dsRNA alone was responsible for the interference effect. Specificity had already been established by work done in Craig's laboratory and others, but it was confirmed in this study by the targeting of a variety of maternally and zygotically expressed genes. In all cases but one, a phenotype consistent with loss of function of only the targeted gene was observed. The one exception was probably due to disruption of closely related homologs, to produce a more severe phenotype than that of the mutant. Finally, in an effort to determine the extent of cell types amenable to interference by dsRNA (and the effects of diluting the injected material by multiple rounds of cell divisions), experiments were designed that targeted expression of green fluorescent protein in a transgenic line constructed by Steve Kostas. The results demonstrated that not all cells are equally susceptible to RNAi and that the effect could be lost owing to dilution of the injected material.

The work published in the 1998 *Nature* paper established that dsRNA was capable of interfering with a gene's activity in a very sequence-specific manner and at substoichiometric abundance with respect to the target mRNA. There were several possible mechanisms that could explain these results, which were not mutually exclusive: the dsRNA was interfering with expression at the level of the gene, or interference involved a catalytic or amplification component.

Evidence supporting all three mechanisms emerged in the following years, as RNAi was found to be a cellular process shared by a wide range of eukaryotes, including the plants and fungi where cosuppression was first described[9,10]. We now know that RNAi generally functions at a post-transcriptional level, resulting in the degradation of mRNAs containing sequences that match the dsRNA; but recent work indicates that, under some circumstances, RNAi in *C. elegans* can lead to a form of transcriptional gene silencing that most probably involves chromatin remodeling[11,12]. It has been firmly established that RNAi-directed processes are essential to maintenance of certain chromatin states in other organisms[13] and can lead to transcriptional gene silencing in plants via DNA methylation[14,15].

However, the mechanistic details that rapidly emerged from a whirlwind of molecular, genetic and biochemical studies published within just a few years of the 1998 study (see the October 2005 issue of *FEBS Letters* for an exhaustive collection of reviews) support the following model. An RNase III–like enzyme called Dicer cleaves the dsRNA (which in the original worm studies was typically several hundred nucleotides long) into small interfering RNAs (siRNAs) approximately 23 nucleotides in length, each of which can recruit an RNA-induced silencing complex (RISC). The siRNA is unwound and the antisense strand becomes available to base-pair with cognate RNAs in the cell. When it does so, an enzyme in the complex (affectionately nicknamed 'Slicer') cleaves the bound mRNA, and the resulting two halves are then rapidly degraded by the cell's RNA-surveillance machinery[16,17]. In addition, even more siRNAs may be generated secondarily through the activity of RNA-dependent RNA polymerases[18].

A tremendous wealth of information has

emerged to reveal a far more complex RNA world than was our understanding a decade ago. A new class of small regulatory RNAs, including the microRNAs, is now recognized as having a far more prominent role in regulating gene activity than previously appreciated[19,20]. RNAi-based mechanisms are also thought to protect the cell against certain viruses and transposable elements that 'expose themselves' through a dsRNA replication intermediate[21,22]. It seems that after this defense mechanism evolved, it was co-opted for regulation of endogenous genes.

Yet questions remain, and new studies generate additional ones. Which aspects of RNAi are evolutionarily conserved (and thus widely shared) processes, and which are more derived (and thus more species specific)? What is the relationship between post-transcriptional and transcriptional mechanisms of silencing, and between heritable and transient forms? Are there definable conditions that will lead to one or the other? An even broader question is just how complex and intertwining the various pathways for processing and regulating RNAs are. How do cells regulate competition between the pathways for processing the microRNAs and endogenous siRNAs that regulate endogenous genes, and the pathway for producing the 'exogenous siRNAs' presumably involved in self-defense mechanisms? How does the cell distinguish among the various small regulatory RNA pathways—some of which result in degradation of the mRNA, others in stability yet translational repression of the mRNA, and yet others in sequestration of the mRNA? Stay tuned.


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