What's new about RNAi?

Meeting on siRNAs and miRNAs

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This Keystone meeting on siRNAs and miRNAs took place in Keystone, Colorado, USA, between April 14 and 19, 2004 and was organized by T. Tuschl and V. Ambros.

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Introduction

RNAi stands for 'RNA interference', which is the silencing of gene expression by the administration of double-stranded RNA (dsRNA). Endogenous RNAi seems to be a primitive sort of immune system, the aim of which is to defend genomes against molecular parasites such as viruses and transposons. During the process of RNAi, the dsRNA is converted into a shorter form: siRNA. The term siRNA stands for 'short interfering RNA', and synthetic versions of these 21-nucleotide molecules are widely used to induce RNAi in mammalian cell systems because they circumvent the aspecific interferon response of these cells to dsRNA. Then there is another species of small RNA molecule, the so-called miRNA (micro RNA). It is important to note that miRNAs are always encoded by the genome itself as hairpin structures, whereas siRNAs can be both artificial and endogenous (Hamilton & Baulcombe, 1999; Aravin *et al*, 2001; Reinhart & Bartel, 2002; Ambros *et al*, 2003). Both molecules feed largely into one and the same process that can either lead to messenger RNA degradation or to the inhibition of protein synthesis. As a rule, siRNAs cause mRNA destruction, whereas miRNAs can cause both. In plants, the majority of miRNAs direct cleavage, whereas miRNAs in animals most often induce translation inhibition; however, examples of translation inhibition in plants and cleavage in animals have been

found (Chen, 2004; Yekta *et al*, 2004). In this review, we follow the fate of these small molecules from their point of synthesis to the execution of their repressive effect (Fig 1) and the use of that effect in the lab. While doing this, we highlight the novel findings that were presented at this Keystone meeting.

Synthesis of siRNAs and miRNAs

The synthesis of siRNAs often occurs *in vitro*, so there is not much interesting biology to discuss here, apart from the fact that the mechanisms of dsRNA uptake and spreading in various organisms (for example, *Caenorhabditis elegans* and plants) are still not completely understood. The siRNAs that are made in the cell itself are derived from dsRNA molecules that are most probably a product of read-through transcription or fold-back structures (V. Ambros, Hanover, NH, USA; Sijen & Plasterk, 2003). How and where these dsRNA molecules are made and processed is not known.

More is known about the production of miRNAs: miRNA genes are transcribed by RNA polymerase II and transcripts are subsequently also capped, spliced and poly-adenylated, as reported by N. Kim (Seoul, S. Korea) and P. Sharp (Boston, MA, USA). Therefore, expression patterns of miRNAs in *C. elegans* can be easily determined by fusing green fluorescent protein to upstream sequences (Johnson *et al*, 2003; Johnston & Hobert, 2003). The nascent transcript of the miRNA is named pri-miRNA (primary miRNA) and can contain more than one miRNA. The individual miRNA-containing hairpin precursor (or pre-miRNA) is excised from this pri-miRNA by the enzyme Drosha (Lee *et al*, 2003) in the nucleus and is assisted by a dsRNA-binding protein, gripper (G. Hannon, Cold Spring Harbor, NY, USA). Drosha is an animal-specific RNase III enzyme and is essential for the production of miRNA precursor structures that can be exported from the nucleus. In plants, this role appears to be taken by one of the Dicer homologues (DCL1; Park *et al*, 2002; Reinhart *et al*, 2002; Xie *et al*, 2004).

The pre-miRNA is then exported to the cytosol (Yi *et al*, 2003; Bohnsack *et al*, 2004; Lund *et al*, 2004), where it is further processed by Dicer (Grishok *et al*, 2001; Hutvagner *et al*, 2001; Ketting *et al*, 2001). This enzyme can take any dsRNA and convert it to si/miRNAs (Bernstein *et al*, 2001), and there have been many models for how this is achieved. However, it now seems clear that the human Dicer enzyme carries out this task by binding, as a monomer, to one end of the dsRNA through its PAZ (Piwi–Argonaute–Zwille) domain (Lingel *et al*, 2003; Song *et al*, 2003; Yan *et al*, 2003), which seems to specifically recognize dsRNA ends produced by RNase III enzymes (Ma *et al*, 2004). This

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positions the two RNase III domains of the Dicer monomer such that they form one active site approximately 21 base pairs away (Zhang *et al*, 2004). In the case of miRNAs, this mode of action leads to the production of only one miRNA of specific sequence, as only the paired end of the pre-miRNA hairpin can be recognized.

Building up towards RISC activity

Biochemistry. The complex that is ultimately responsible for silencing has been named the RNA-induced silencing complex (RISC), which incorporates both siRNAs and miRNAs. Only single-stranded RNA is incorporated, however, and which of the two strands makes it into RISC is determined by the thermodynamically asymmetric nature of the siRNA: the strand with the most loosely base paired 5' end is the lucky one (Khvorova *et al*, 2003; Schwarz *et al*, 2003). P. Zamore (Worcester, MA, USA) reported that this asymmetry is sensed by Dicer in complex with the dsRNA-binding protein R2D2, which literally takes this strand to the RISC complex (Lee *et al*, 2004; Pham *et al*, 2004; Tomari *et al*, 2004). What happens next is most likely determined by a combination of factors: the origin of the small RNA (that is, whether it has been processed by Drosha and/or Dicer), associated proteins and the extent of base pairing between the target mRNA and the si/miRNA.

One of the outcomes is cleavage of the mRNA. The protein that executes this cleavage ('slicer') remains elusive, but it is now known what chemistry this enzyme uses: a 3' hydroxyl and a 5' phosphate group characterize the cleavage product (Martinez & Tuschl, 2004; Schwarz *et al*, 2004). Also, RISC behaves like a true enzyme, so it catalyses many rounds of cleavage.

The other outcome—translation inhibition—is even less understood. What step of translation is actually inhibited: initiation or elongation? Or is the process of translation not inhibited at all? Obviously, as the outcome is an absence of protein production, the latter hypothesis would imply a scenario in which miRNAs trigger protein degradation. In short, we do not know. However, Sharp presented interesting data that suggest that one way of translational silencing might indeed involve nascent chain degradation.

Genetics. RNAi was first firmly established in *C. elegans* (Fire *et al*, 1998), and it is clear that the role for this organism in the study of RNAi-related processes is not yet over. A number of *C. elegans* screens were described at the meeting, which identified factors involved in RNAi or miRNA function (G. Ruvkun, Boston, MA, USA; C. Mello, Worcester, MA, USA; R. Plasterk, Utrecht, The Netherlands). How these factors fit in the current biochemical framework is not clear, but more links between biochemical assays and genetic experiments are being generated (Lee *et al*, 2004; F. Slack, New Haven, CT, USA; Mello; Plasterk).

One family of proteins that is central to the processes discussed here is the Argonaute family. These proteins have been implicated in various forms of gene silencing and are found physically associated with miRNAs and siRNAs. They seem to have an important role in the structural and maybe even catalytic features of the RISC enzyme and are now being systematically analysed both in worms, in which more than 25 family members have been identified (Mello), and in mice, which contain six family members (Hannon). Some of these proteins are essential for viability and fertility, which indicates again

Fig 1 | Schematic representation of miRNA and siRNA mediated silencing. As little is known about the synthesis of endogenous siRNAs, this has been omitted from the figure. RISC, RNA-induced silencing complex.

that RNAi-like processes are not only useful tools for the experimentalists but also involved in crucial biological processes.

Target identification

The identification of novel miRNA genes is thought to be around 80% complete in *C. elegans*, although new ones are still being identified through genetics, for example *lsy-6* (Johnston & Hobert, 2003). The human genome probably encodes around 250 miRNAs, most of which have already been detected; however, detailed analysis of specific tissues can still result in the identification of new miRNAs. For instance, M. Stoffel (New York, NY, USA) reported the identification of ten pancreatic-islet-specific miRNAs. With miRNA gene discovery nearing saturation, one of the most prevalent research activities entails the next step, which is the identification of relevant miRNA–mRNA pairs; in other words, which miRNA controls the expression of which mRNA. In plants, this is an easier task than in animals, as in plants the fraction of (nearly) perfect miRNA–mRNA matches is much higher. Nevertheless, in both kingdoms, miRNAs seem to contain a ‘seed’ sequence: positions 2–8 most often display perfect pairing to the mRNA (Lewis *et al*, 2003; Stark *et al*, 2003), and these positions are also very sensitive to mutations (Lewis *et al*, 2003; Doench & Sharp, 2004; J. Brennecke, Heidelberg, Germany; D. Bartel, Boston, MA, USA; Zamore; Plasterk). Using computational approaches, valid targets can be identified (Lewis *et al*, 2003; Stark *et al*, 2003; Bohnsack *et al*, 2004; Rajewsky & Succi, 2004), although the substantial background noise makes verification through experimentation essential. In this light, it is interesting to note that good old genetics is also resulting in the identification of miRNA–mRNA pairs (Brennecke *et al*, 2003; Johnston & Hobert, 2003; A. Rougvié, Minneapolis, MN, USA; O. Hobert, New York, NY, USA). Altogether, a picture is emerging that for any given miRNA there will be not one but several target mRNAs. Given the high number of miRNAs, these findings have led to the speculation that more than 10% of the genome will be regulated through the action of miRNAs (D. Marks, Boston, MA; Bartel; Brennecke).

Biological effects of miRNA knockdown

What is the effect of miRNA depletion? This is an interesting question, but one that is hard to address using reverse genetics, as the target to be mutated is very small. Nevertheless, a significant part of all miRNA genes in *C. elegans* have been knocked out (R. Horvitz, Boston, MA, USA; Ambros; Bartel). Despite this, few abnormal phenotypes have been detected, which indicates that redundancy might be an important issue in studying miRNA function. As an alternative, 2′O-methylated RNA oligos that are complementary to miRNA sequences, and thus inhibit their function by mimicking a target mRNA, have been shown to be effective at depleting specific miRNAs *in vitro* (*Drosophila* extracts) and *in vivo* (*C. elegans*; Hutvagner *et al*, 2004). This technology is now also being applied to other organisms *in vivo* such as *Drosophila* (U. Gaul, New York, NY, USA), in which up to 50% of the injected 2′O-methyl oligos resulted in detectable phenotypes. Such an approach is also viable in vertebrates, as shown by a specific inhibition of *let-7* in the zebrafish (Plasterk).

Applications

No matter how all this may work, it is clear that RNAi is still gaining popularity as a tool for knockout analysis of genes on a genome-wide scale. Until recently, these screens were the domain of

researchers working on worms and flies, but now libraries for scanning the human genome have been described and made available (Berns *et al*, 2004; Paddison *et al*, 2004). The main issue about such libraries remains the degree of (wanted) on-target effectiveness versus (unwanted) off-target effects. The discussion about the relevance of off-target effects has not yet settled, but it is clear that recent insights into the mechanisms of RNAi have already made it to the siRNA design table with positive effects on the specificity of these molecules. How big or small these specificity problems are in practice remains to be seen, but screens so far indicate that off-target effects are not a major problem in the daily use of such libraries in the laboratory (Berns *et al*, 2004; Paddison *et al*, 2004).

To make things work even better, many companies are testing chemical modifications of siRNAs. They all reported success at the meeting, but we will have to exert patience for the disclosure of the identities of these modifications. The results are promising: the effective concentration of siRNAs could be significantly reduced and the off-target effects were lower. Such gains in effectiveness are obviously required if siRNAs are ever going to make it into the clinic. It is clear, from a fundamental point of view, that RNAi could be an effective strategy for the treatment of viral infections such as respiratory syndrome virus (S. Barik, Mobile, AL, USA) and hepatitis (D. Morrissey, Boulder, CO, USA; M. Kay, Stanford, CA, USA), provided the problems of stability and delivery can be tackled. An alternative method of delivery might be the use of clinically suitable viral vectors. These can be modified to deliver siRNAs that are effective at turning off viral genes in animals, and these may find their way into clinical trials soon (Kay).

Conclusion

The field of RNAi is rapidly coming of age. Some of the mechanistic insights have already reached the level of atomic resolution; however, for other aspects of the process, not even the exact molecular effects are known. The number of labs working on the phenomenon is increasing as RNAi-related processes keep appearing in all sorts of biological contexts, which is making progress in the field very rapid indeed. From the meeting in Keystone it became clear that much good can still be expected from these studies—so, stay tuned.

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