CONTENT ***The 90th Boehringer Ingelheim Fonds International Titisee Conference »RNA silencing« took place between 21 and 24 October 2004 and was organized by Thomas Jenuwein, Research Institute of Molecular Pathology (IMP), Vienna, Austria, and Thomas Tuschl, The Rockefeller University, New York, NY, USA. The meeting brought together a diverse spectrum of scientists concerned with understanding the function of double-stranded RNA triggering gene silencing in animals, plants, and fission yeast. The silencing processes are guided by processing products of the dsRNA trigger, which are known as small interfering RNAs (siRNAs) and microRNAs (miRNAs). The presentations at the conference discussed the natural function of these small RNAs and the possibility to exploit RNA silencing mechanisms to mediate silencing of disease-causing genes.***

RNA silencing

90th International Titisee Conference

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• RNA silencing or RNA interference (RNAi) refers to the process of sequence-specific regulation of gene expression triggered by double-stranded RNA (dsRNA)[1]. RNA silencing mechanisms are conserved in almost all eukaryotes[2]. Some of the natural functions of RNA silencing processes are well understood, while others remain elusive. Depending on the organism and the source of the dsRNA trigger, RNA silencing processes mediate transcriptional and/or post-transcriptional gene silencing. Post-transcriptional RNA silencing mechanisms are defined by either mRNA degradation or translational repression (for review see reference 3). Effective target mRNA degradation requires extensive sequence complementarity between the dsRNA trigger and target RNA. On the other hand, in certain evolutionarily conserved contexts, imperfect complementarity leads to effective translational repression without extensive target RNA degradation[4].

RNA silencing is important for the regulation of development and the control of transposition events in both plants, where RNA silencing was first observed, and in animals[3,5,6]. RNA silencing also plays an antiviral role in plants and in insects such as *Drosophila melanogaster*[7]. In fission yeast, RNA silencing is one of the systems that establishes and maintains the heterochromatin structure of the centromere and mating type locus[8]. Chromatin modifications are carried out by histone modifying enzyme complexes, some of which are RNA-dependent.

In vertebrates and mammals, longer dsRNA (> 30 base pairs) elicits a potent sequence-unspecific interferon response that globally interferes with gene expression[9], and which made it difficult to initially observe gene-specific RNA silencing mechanisms. During RNA silencing, longer dsRNA is processed to about 21-nucleotide short interfering RNAs (siRNAs), which when introduced artificially into mammalian cells do not generally trigger this global unspecific response[10].

The aim of this conference was to bring together the fields of RNA silencing and chromatin modification and to explore the regulatory role of dsRNA. The mechanisms that control the establishment of open or closed chromatin environments (also known as euchromatin and heterochromatin respectively) are under close investigation, and dsRNA has been established as an important trigger for heterochromatin formation in fission yeast and in plants. The conference provided an in-depth overview of the chromatin structure and dynamics, as well as of the general role of RNA in driving chromatin changes, the mechanisms of RNA silencing, the natural sources of dsRNAs, and the exploitation of the cellular RNA silencing machinery for technological and therapeutic purposes.

Biochemical aspects of the mechanisms of RNA silencing

• A graphic representation of the RNA silencing processes and its molecular steps is provided in *figure 1*. Processing of dsRNAs to siRNAs involves the ribonuclease (RNase) III Dicer. Witold Filipowicz biochemically characterized mammalian Dicer. He showed that previous models of the active sites needed to be revised

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and that Dicer contains two single active sites, each of which cleaving one strand of the dsRNA backbone(11).

Following siRNA generation, one of the siRNA strands is incorporated into a complex containing a member of the Argonaute (Ago) protein family. The complex, depending on the specific Ago protein, is either able to cleave complementary target mRNAs, e.g. AGO2 (also known as EIF2C2) in human(12,13), or is thought to repress translation. Witold Filipowicz presented an experimental approach in support of the role of Ago proteins as translational repressors. By tethering Ago protein fusions to the 3' untranslated region (UTR) of a reporter gene, he could show that they repressed translation, while control protein fusions had no effect(15). Dinshaw Patel presented a crystal structure of the PAZ domain (named after its presence in Piwi, Argonaute, and Zwille proteins) of Ago1 which specifically recognizes the 2-nucleotide (nt) 3' overhang of duplex siRNAs(1). He also revealed the structure of the full-length bacterial Argonaute of *Aquifex aeolicus* and proposed a stereochmically robust model of how the double-stranded miRNA/target RNA complex could be placed into the protein.

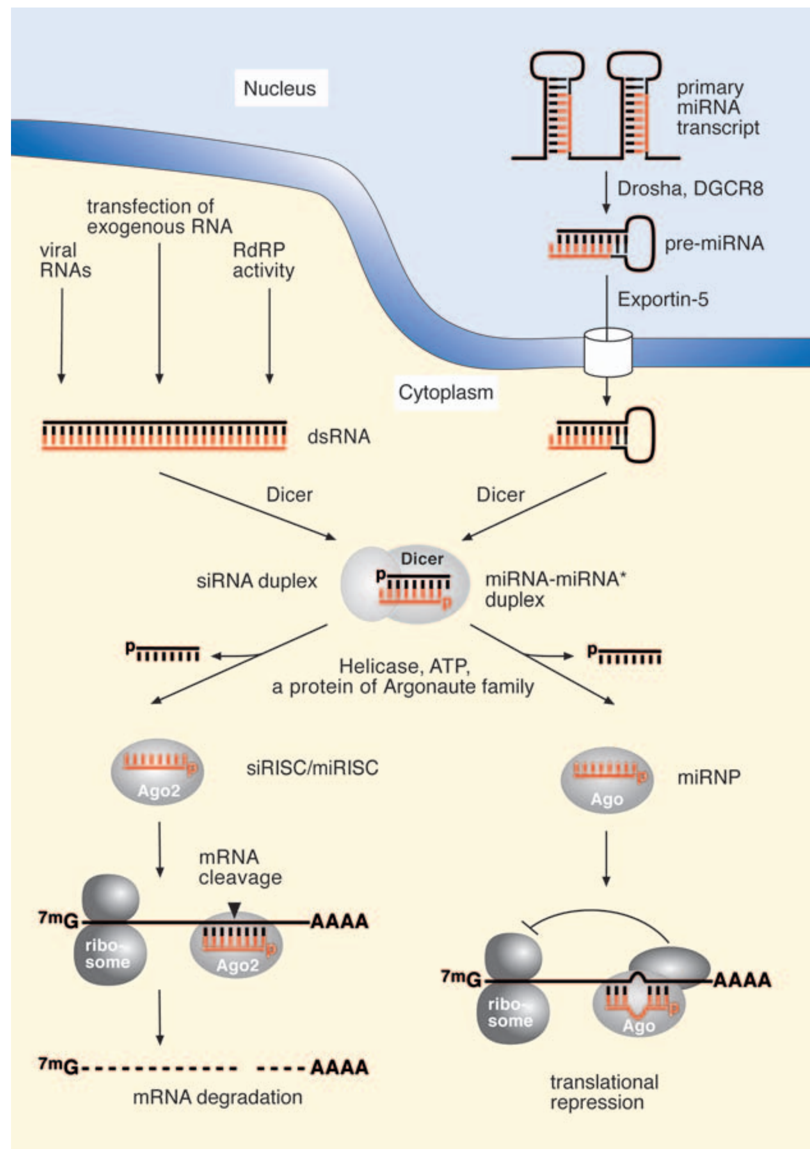

FIG. 1: Model of post-transcriptional gene silencing pathways guided by small RNAs. miRNA primary transcripts (pri-miRNA) are processed to miRNA precursors (pre-miRNA) by Drosha RNase III and its interacting partner, DGCR8. The pre-miRNA is subsequently exported to the cytoplasm using the export receptor exportin-5 and processed by Dicer RNase III to a »siRNA-like« miRNA-miRNA duplex intermediate. The single-stranded miRNA is loaded onto a member of the Ago protein family and the miRNA* is degraded. miRNPs associate with partially complementary sequences in the 3' UTR to inhibit translation. dsRNA may also be of viral origin, artificially introduced, or generated by RdRP. Both siRNAs and miRNAs can be assembled into Ago2-containing RISC complexes (siRISC, miRISC). Ago2 in mammals mediates the cleavage of complementary targets. (7mG: 7-methyl guanosine; AAAA: poly-adenosine tail; p: 5' phosphate)*

David Baulcombe described the characterization of a new RNA polymerase involved in the silencing of endogenous genes in *Arabidopsis thaliana.* His laboratory had already identified the *sde4* (silencing-defective 4) mutant, which was defective in siRNA production and methylation of a retroelement, and which also showed a partial loss of transgene silencing. The characterization of the *sde4* mutation revealed that plants encode subunits for a fourth RNA polymerase (pol IV) in addition to the known DNA-dependent RNA polymerases I, II, and III. Mutational analyses of the two largest subunits showed that pol IV silences certain transposons and repetitive DNA involving RNA-dependent RNA polymerase Rdr2 and RNase III Dcl3(16). A function of pol IV in RNA silencing might provide one explanation for the contradictory observation that chromatin silencing is dependent on RNA transcription. Pol IV could be resistant to DNA or chromatin modifications affecting RNA polymerases I through III, and might therefore stably maintain the silenced state.

Small RNAs in heterochromatin formation and maintenance

• Genetic information is encoded in linear sequences of DNA, which is packaged inside the cell in chromatin complexes. The structure of the chromatin of a specific gene determines if it is repressed or amenable to expres-

sion. David Allis reviewed mechanisms of establishment of heterochromatin and transcriptional silencing. He concentrated on histones H3 and H4, which are highly conserved in all eukaryotes. The status of methylation of conserved lysine residues (K) and phosphorylation of serine (S) of H3 and the readout of these modifications by proteins define both the open and repressed chromatin state[17]. The proteins responsible for recognizing H3-K9 methylation sites are chromodomain-containing proteins (e.g. HP1, Pc), which trigger heterochromatin formation. In species such as *Schizosaccharomyces pombe* or *A. thaliana*, the RNAi machinery and small RNAs derived from repetitive sequences are required for H3-K9 methylation and transcriptional gene silencing of repeat regions[18,19].

The genetically and biochemically best characterized RNA silencing process related to heterochromatin establishment and maintenance is centromeric gene silencing in *S. pombe*, which was described by Shiv Grewal (*Figure 2*). His group, in collaboration with Danesh Moazed, had earlier discovered a complex containing small RNAs, ago1, a novel protein tas3, and the chromodomain protein 1 (chp1). This complex is required for establishing silenced heterochromatin at centromeric regions and has therefore been named RNA-induced transcriptional silencing (RITS) complex[20]. The RNase III Dcr1 was also required for establishing heterochromatin, but not for maintaining its structure. In mutants of the chp1 chromodomain or of the histone methyl transferase clr4 (cryptic loci regulator 4) siRNAs are lost, since RITS no longer co-localizes with methylated H3-K9 heterochromatin. This might suggest that siRNAs can only be captured near chromatin, i.e. at the site of dsRNA synthesis. Indeed, Grewal showed that the RNA-dependent RNA polymerase rdp1 interacted with RITS, presumably to establish a self-enforcing loop for the amplification of the silencing trigger. rdp1 was only recently shown to be part of a trimeric complex termed RNA-directed RNA polymerase complex (RDRC)[21] This complex contains two conserved proteins, hrr1 (helicase required for RNAi-mediated heterochromatin assembly 1) an RNA helicase, and cid12 (caffeine-induced death resistant 12), a member of the polyA polymerase family[22]. Although the function of these two proteins has not yet been established, Craig Mello surprised us by recounting that he too had identified a protein, termed rde-3 (RNAi deficient 3), in the nematode worm *Caenorhabditis elegans*, which was closely related to cid12[21]. The rde-3 is required for siRNA accumulation and for efficient RNAi in all tissues. Its nucleotidyl transferase activity may be required to tag the targeted mRNA for serving as a template for the RNA-dependent RNA polymerase to specifically feed into the amplification process of the trigger dsRNA.

One of the most striking forms of RNAi-related gene silencing was observed in the ciliate *Tetrahymena thermophila*. During sexual conjugation and the ensuing developmental process, this organism forms a germline micronucleus and a somatic macronucleus from a zygotic nucleus. The macronucleus is generated by coordinated elimination of about 6,000 intervening eliminated sequences (IESs) specific to the micronucleus. A scanning model for IES elimination has been proposed: in early conjugation, dsRNA transcripts that emerge from the micronucleus are processed to small, approximately 28-nt RNAs. These are transferred to the old macronucleus, where any small RNAs homologous to the macronucleus are destroyed. The remaining small RNAs, which are cognate to IESs of the micronucleus, are then transferred to the developing new macronucleus, where they target the IESs for elimination. The Ago member protein Twi1p (Tetrahymena piwi-related protein 1) is required for small RNA accumulation and IES elimination. Twi1p first localizes to the parent macronucleus and then to the new macronucleus[23]. During elimination, the IES are H3-K9-methylated and are found in dense, heterochromatin-like regions. Two chromodomain-containing proteins, Pdd1p and Pdd3p (programmed DNA deletion protein 1), were shown to bind methylated H3-K9, and are necessary for DNA elimination. Unlike other species, where the endpoint of silencing is the formation of heterochromatin, the ciliate takes gene silencing one step further and goes on to eliminate the heterochromatin sequences.

Gunter Reuter and Thomas Jenuwein described the enzymes that methylate histones. Initially identified in genetic screens for suppressors of position-effect variegation of *D. melanogaster* eye pigmentation, the Su(var)3-9 was shown to be the H3-K9 methyl transferase (for review see reference 24). Further specific histone methyl transferases were also described[25]. In mammals, about 50 different proteins with a conserved methylase motif (or SET domain) have been identified (for review see reference 26). Histone methyl transferases can mono-, di- and tri-methylate lysine residues, and generation of specific antibodies that can distinguish between the different methylation substrates and the degree of methylation have become crucial to an understanding of heterochromatin state and function[27]. Thomas Jenuwein further presented the profile of repeat-associated histone lysine methylation states in the mouse epigenome[28]. These data revealed selective enrichment of distinct H3-K9, H3-K27, and H4-K20 methylation marks across tandem repeats (e.g. major and minor satellites), DNA transposons, retrotransposons, long interspersed nucleotide elements, and short interspersed nucleotide elements. To what extent these methylation marks are linked to dsRNA transcripts remains to be evaluated in mammals.

Further connections between RNA and heterochromatin in animals

- Peter Becker reported on the characterization of the roX1/2 RNAs (RNA on X) required for dosage compensation of genes located on the single

male X chromosome in relation to their counterparts on the two female X chromosomes (for review see reference 29) in *D. melanogaster*. These large non-coding RNAs (3.2 and 0.8 kb respectively) are incorporated into the dosage compensation complex (DCC) that also contains the proteins Mle (maleless), Msl1, Msl2, Msl3 (male-specific lethal 1-3), and the histone acetyltransferase Mof (males-absent on the first protein). Both Mof and Msl3 carry RNA-binding chromo-related domains (which do not bind me-H3-K9). These domains are thought to be important for the interaction with roX RNAs, albeit they are not the primary determinants for targeting mof to its X chromosome territory[30].

Renato Paro described the role of intergenic transcripts important for homeotic gene silencing during development of *D. melanogaster*[31]. The Polycomb and Trithorax group (PcG and TrxG) genes are important in establishing cellular memory during development (for review see reference 32). PcG proteins act as repressors of several hundred genes and bind to defined sites, referred to as polycomb responsive elements (PREs). Transcription through PREs is an important signal for triggering PcG binding and silencing of the adjacent gene.

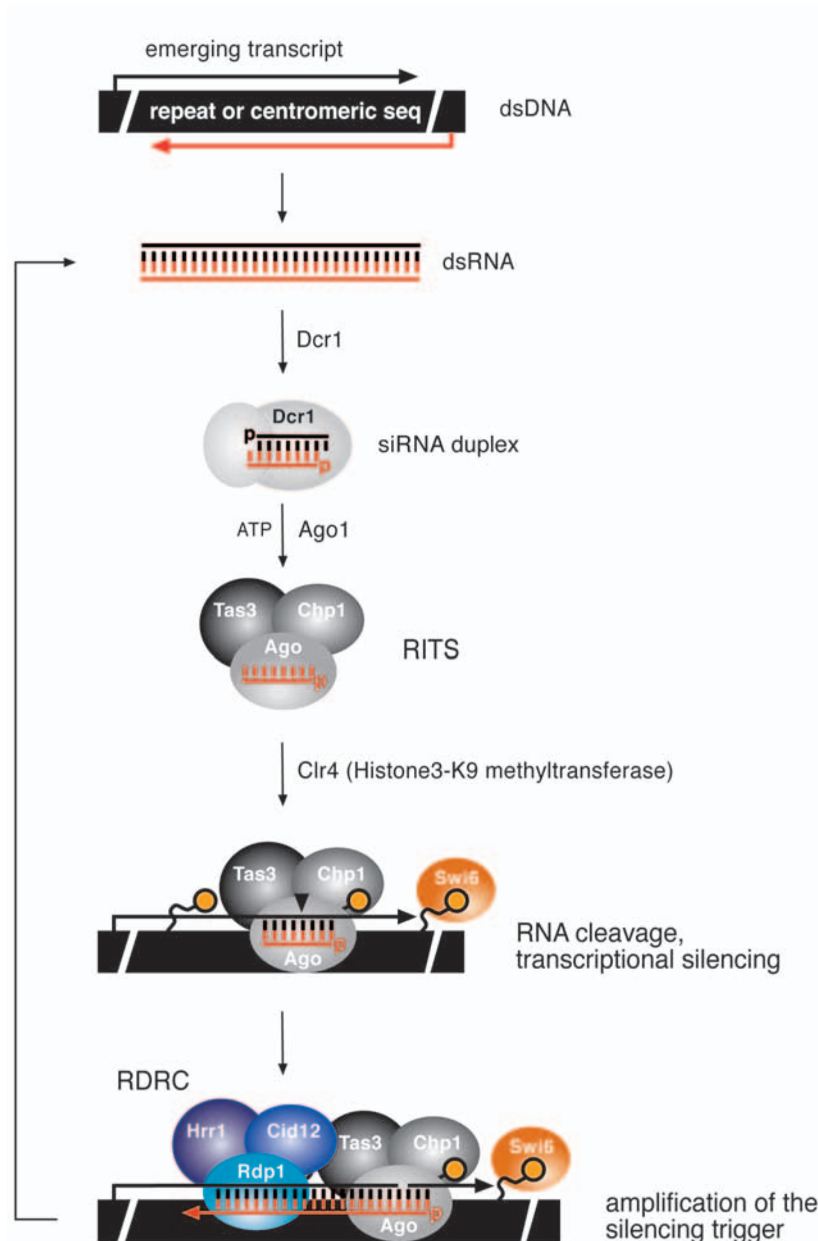

***FIG. 2:** Model of small RNA-guided transcriptional silencing in* S. pombe*. Sense and antisense strands of repeat-associated sequences give rise to dsRNA. Dicer (Dcr1) generates short double-stranded repeat-associated siRNA (rasiRNA) that are assembled into RITS complexes, which guide it to chromatin and recruit the Histone3-K9 methyltransferase Clr4. RITS contains Ago1, the Chp1 chromodomain protein, and Tas3[30]. Methylation of Histone3-K9 recruits the heterochromatin protein Swi6, which establishes heterochromatin. RITS can also mediate cleavage of transcripts emerging from repetitive elements and recruit RDRC, which contains a member of the RNA-dependent RNA polymerase, Rdp1. Rdp1 has two conserved proteins, Hrr1, an RNA helicase, and Cid12, a member of the polyA polymerase family[31]. The newly synthesized dsRNA amplifies the silencing process and maintains the repressed chromatin state.*

MicroRNAs

• MicroRNAs (miRNAs) are non-coding, small regulatory RNA molecules. They are about 22 nt long, and of evolutionarily conserved sequence (for review see reference 33). miRNAs are found in animals and plants, and function by repressing translation or by cleaving messenger RNA (mRNA), depending on the level of complementarity of the base-pairing. Because hundreds of miRNAs have now been identified, it is becoming increasingly clear that many, if not the majority of protein-coding genes, are regulated by miRNAs in a cell-type specific or developmental manner.

The miRNAs are processed in a step-by-step fashion from longer primary polymerase II transcripts (pri-miRNAs) that contain one or more short dsRNA hairpin structures (pre-miRNAs) (for review see reference 34). In animals, miRNAs are excised sequentially from pri-miRNAs by two distinct RNase III enzymes. The pri-miRNA is cleaved in the nucleus by the RNase III Drosha to release the pre-miRNA, which is then exported by Exportin-5 to the cytoplasm (for review see reference 35) and further processed by RNase III Dicer to remove the stem-closing hairpin loop.

The product of the reaction is a miRNA duplex composed of the mature 21- to 23-nt miRNA and its complementary 21- to 23-nt strand. The structure of the miRNA duplex is similar to siRNA duplexes, both of which have 2-nt 3' overhangs and 5' phosphates. In the plant *A. thaliana*, where Drosha homologues have not been identified, pri- and pre-miRNAs are generated by a single RNase III, the nuclear localized DICER-LIKE 1 (DCL1)[40]. The miRNA duplex, rather than the hairpin, is presumably transported to the cytoplasm.

In the cytoplasm, the miRNA is incorporated into an Ago protein member containing ribonucleoprotein particle (miRNP) forming an effector complex containing single-stranded mature miRNA[37]. The complementary strand of the miRNA only rarely becomes associated with Ago protein and is generally degraded. Ago proteins occur as a family in most species, and can be subdivided into two subfamilies. One of them contains the more ubiquitously expressed members and the other the more germ line- and stem cell-specific members[38]. All the ubiquitously expressed members are associated with siRNAs or miRNAs[12,13]. However, only one of the ubiquitously expressed members (Ago2 in humans and in *D. melanogaster*) is associated with cleavage activity[12,14]; its small RNA-loaded complex is called RNA-induced silencing complex or RISC[39]. Depending on the type of small RNA associated with Ago2, one also distinguishes between miRISC and siRISC. In mammals, the function of the other ubiquitously expressed Ago members is still unresolved. Since they are unable to guide cleavage, they possibly function as translational regulators.

At the conference, the miRNA pioneers and representatives for all relevant model organisms expressing miRNAs made presentations. The first miRNA gene, *lin-4 (lineage-deficient 4),* was originally identified (genetically) in *C. elegans*[40], but was not characterized until several years later[41]. Victor Ambros, who first characterized *lin-4*, described the regulatory networks involving lin-4 and let-7 (lethal 7) miRNAs and their targets and what impact this regulation has on developmental timing. These two miRNAs mediate translational regulation of numerous genes, including *lin-14*, *lin-28,* and *lin-41* (for review see reference 42 and *table 1*). His laboratory is involved in a large-scale project to knock out more than 70 miRNA genes in *C. elegans*. Pre-

miRNA	Species[a]	Function of miRNA	Targets	Role of target	References
lin-4	Ce	Developmental timing	*lin-14* *lin-28*	probable transcription factor Cold shock domain protein	Wightman, B et al. (1993) *Cell 75* Moss, EG et al. (1997) *Cell 88*
let-7	Ce	Developmental timing	*lin-41* *hbl-1* *daf-12* *pha-4* *lss-4* *die-1* *let-60*	RNA-binding protein Transcription factor Transcription factor Transcription factor Transcription factor Transcription factor Signal transduction	Abrahante, JE et al. (2003) *Dev. Cell 4* Grosshans, H et al. (2005) *Dev. Cell 8* Johnson, SM et al. (2005) *Cell 120* Lin, SY et al. (2003) *Dev. Cell 4* Slack, FJ et al. (2000) *Mol. Cell 4*
let-7 family	Hs	Downregulated in lung cancer	*ras*	Signal transduction	Johnson, SM et al. (2005) *Cell 120*
lsy-6	Ce	Neuronal cell fate	*cog-1*	Transcription factor	Johnston, RJ et al. (2003) *Nature 426*
miR-273	Ce	Neuronal cell fate	*die-1*	Transcription factor	Chang, S et al. (2004) *Nature 430*
bantam	Dm	Cell death, proliferation	*hid*	Pro-apoptotic protein	Brennecke, J et al. (2003) *Cell 113*
miR-14	Dm	Cell death, fat storage	unknown	unknown	Xu, P et al. (2003) *Curr. Biol. 13*
miR-181	Mm	Haematopoietic cell fate	unknown	unknown	Chen, CZ et al. (2004) *Science 303* Chen, CZ et al. (2005) *Semin. Immunol. 17*
miR-375	Mm	Insulin secretion	*mtpn*	Vesicle transport	Poy, MN et al. (2004) *Nature 432*

TAB. 1: Animal miRNAs and their targets and function (Ce: C. elegans; Dm: D. melanogaster; Hs: H. sapiens; Mm: M. musculus).

liminary analysis and the absence of drastic phenotypes in the deletion mutants suggested strong redundancy in the functions of miRNAs. For example, single member deletion of the let-7 miRNA family (let-7, miR-48, 84, or 241) was lethal only for let-7, while individual knockout of the other members had little effect. However, the knockout of all three weak phenotype members resulted in lethality. It is therefore important to consider miRNA sequence relationships and partial redundancy when examining the function of individual miRNAs.

Bioinformatic approaches to identify miRNAs and their targets

• In the past few years, several hundred miRNAs have been identified by direct cloning strategies or bioinformatics approaches in plants, *C. elegans, D. melanogaster,* zebrafish, mouse, rat, and human (for review see reference 33). However, the experimental and computationally predicted numbers vary greatly, depending on the research groups and the strategies chosen. The current number of miRNAs in invertebrates defined experimentally is close to 100, increasing to about 250 in mammals (www.sanger.ac.uk/Software/Rfam/mirna/). Ronald Plasterk presented a miRNA gene identification and prediction approach using phylogenetic »shadowing«[43]. He identified 979 candidate miRNAs by comparing the genomes of human/mouse and human/rat. 678 of these were conserved in zebrafish and chicken. About 20% of the predictions could be experimentally confirmed. The remaining candidates have probably not yet been detected because they are only expressed in specific and rare tissue types, or they are false-positive predictions.

Candidate targets of plant and animal miRNAs have now been computationally predicted by many different groups, often with minimal overlap and little experimental validation (for review see reference 33). Target prediction algorithms rely heavily on the few genetically identified and experimentally validated interactions of let-7 and lin-4 with their target mRNAs in *C. elegans*. Furthermore, sequence alignments of closely related miRNAs usually show variations near the middle or 3' end, indicating that conservation of the 5' residues (position 2 to 8) is most critical for function[44]. Using transgenic reporter genes in *D. melanogaster*, Stephen Cohen proposed three distinct classes of miRNA-target RNA interactions[45]. The »canonical« binding site was characterized by perfect binding of positions 2-8, a short internal loop or bulge, and pairing of the 3' end of the miRNA. This type of interaction occurred between Hairy (H) and miR-7. A second type of interaction, called »seed only«, merely required the binding of positions 2 to 8, as represented by the Bearded (Brd) mRNA and miR-4. The last type of interaction, »3' compensatory«, is defined by imperfect pairing in the seed nucleotides 2 to 8. The imperfect pairing is compensated by strong hybridization in the 3' end, and is exemplified by the interaction between Sex combs reduced (Src) and miR-10. According to his estimates, there are about 80 target mRNAs per miRNA in the fly. Given that we know 78 miRNAs from *D. melanogaster*, nearly half of all protein-coding genes appear to be targeted by miRNAs.

David Bartel presented computational and experimental identification of miRNAs targets in plants and animals. In plants, miRNAs recognize their target mRNAs exclusively by perfect or near-perfect pairing followed by cleavage. The 92 known *A. thaliana* miRNAs constitute 22 miRNA gene families; 18 of these families are conserved in rice. The members of these conserved 18 families share 83 unique targets, 70 of which have developmental function. It is more difficult to predict invertebrate, vertebrate, and mammalian targets, since imperfect pairing interactions are still able to confer miRNA regulation. Furthermore, highly complementary segments of mRNAs to miRNAs are practically absent from animal genomes. Since miRNA sequences are conserved in different species, their target sequences must also be conserved. Such sequence conservation criteria were used to filter seed complementary target-binding sites, and approximately one third of mammalian genes were predicted to be regulated by miRNAs[46].

Chris Sander developed target prediction tools (www.microrna.org), which do not require perfect seed complementarity and which evaluate the pairing of the remainder of the miRNA sequence to the target site. His programme also took evolutionary conservation of predicted sites into consideration, and he came to the conclusion that about 10% of the mammalian genes are targeted by miRNAs[47]. Sander made the interesting observation that mRNA targets of the fragile X syndrome mental retardation protein, FMR, also serve as miRNA targets. This observation supports the recently identified molecular interactions between this protein and miRNA-protein complexes[48].

The function of miRNAs – new insights

• Markus Stoffel presented the first functional characterization of a mammalian miRNA target controlled by the vertebrate-specific miR-375[49]. He isolated this miRNA from the insulin-producing pancreatic β-cells. miR-375 regulates myotrophin, which is important for the exocytosis (secretion) of hormones, including insulin. Overexpression of miR-375 reduced secretion, while inhibition of miR-375 by antisense 2'-O-methyloligoribonucleotides increased secretion. Given the specific expression of this miRNAs in neuroendocrine cells of the pancreas, it is possible that the characterization of the function of this miRNA sheds some light on the causes of diabetes.

Phillip Sharp approached function by first revealing specific expression patterns of miRNAs during mouse embryogenesis. A transgenic mouse that constitutively expressed a β-galactosidase sensor gene with a specific miRNA complementary site in the 3' untranslated region is able to reveal a specific miRNA expression

pattern. Using this approach, the expression pattern of the developmentally regulated miR-1, let-7, miR-10a, and miR-196a[50] was revealed.

Thomas Tuschl reported that miRNA genes are also found in the herpesvirus family, including Epstein-Barr virus, Kaposi sarcoma virus, and cytomegalovirus[51]. Some of these viral miRNAs appear to function as siRNAs, since they are transcribed in opposite orientation to protein-coding viral genes. Others are differentially regulated in lytic and latent stages of the virus and presumably target viral and/or host cell genes. The analysis of miRNA-deficient viruses should provide insights into the role of these new miRNAs.

In the more distant future, animal models deficient in miRNA maturing enzymes or miRNA gene loci will be created to evaluate the biological function of miRNA families or their individual members. It is also anticipated that mutations in miRNAs or their target sequences may be found and linked to human diseases.

Technological and therapeutic applications of RNA silencing

• The siRNAs and short hairpin RNAs (shRNAs) have become a valuable genetic tool. Several research groups already developed expression vectors to direct the synthesis of shRNAs that are continuously processed to siRNAs that stably suppress gene expression. René Bernards presented a study in which shRNA expression vectors, directed against fifty human de-ubiquitinating enzymes, were used to assess the involvement of these enzymes in cancer-relevant pathways. The inhibition of one of the de-ubiquitinating enzymes, the familial cylindromatosis tumour suppressor gene (*CYLD*), resulted in an enhanced activation of the transcription factor NF-κB and an increased resistance to apoptosis. The mechanism is probably the reason why the loss of *CYLD* contributes to oncogenesis[52]. Aspirin derivatives that inhibit NF-κB activity are currently being used in a clinical trial as a therapeutic intervention strategy to restore growth control in patients suffering from familial cylindromatosis. Bernards' laboratory recently constructed a retroviral shRNA expression library. This allows the stable suppression of about 8,000 human genes, and was used to identify novel modifiers of the p53 pathway[53]. In an alternative approach, the shRNA expression vector libraries can also be used in screens undertaken in a polyclonal format. Here the single shRNA expression vector associated with a specific phenotype can be identified by microarray analysis (siRNA bar-code screens). The shRNA expression libraries will greatly facilitate large-scale loss-of-function and synthetic-lethal genetic screens in mammalian cells.

It is apparent that if one was able to introduce siRNAs into specific cell types affected by diseases due to over- or misexpression of a cellular or viral gene, a new gene-specific therapy would be on the horizon (for review see reference 54). To minimize both the unspecific effects of siRNAs and the unwanted targeting of closely related mRNA sequences, Fran Lewitter presented a software package that uses genome-wide information to reduce the selection of problematic sequences from the mRNA due to be targeted[55]. The user is also able to define sequence patterns for the siRNA search, which rely on siRNA design rules developed by various laboratories or based on personal preference. Judy Lieberman discussed different disease-related applications and formulations of siRNAs. Lipid-based formulation of siRNAs can be used to deliver siRNAs to the vaginal epithelium, and targeted delivery of protein-complexed siRNAs towards specific cell surface receptors was explored. These studies made clear that the effective and cell type-specific delivery methods represents the biggest hurdle in many systemic applications of siRNAs in mouse and man. These problems may be solved in the future either by gene therapy-based approaches for the introduction of shRNAs or by developing synthetic, chemically modified siRNAs with enhanced pharmacological properties.

Outlook

• RNA silencing has been shown to be an important and complex gene regulatory mechanism in practically all eukaryotes. The dsRNA trigger can mediate mRNA degradation and translational repression, or initiate and maintain heterochromatin structure, which leads to transcriptional gene silencing of genes embedded in these regions. The biochemical details of these various processes are just being revealed, while the dsRNA-triggered gene silencing technology is already being feverishly exploited for systematic inactivation of gene function in various model organisms or mammalian cell culture. The therapeutic applications are being examined in various academic and industrial circles and one of these days, we can hopefully avail ourselves of small RNAs for the treatment of genetic ailments.

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