The regulation of genes and genomes by small RNAs

Victor Ambros[1],* and Xuemei Chen[2]

A recent Keystone Symposium on 'MicroRNAs and siRNAs: Biological Functions and Mechanisms' was organized by David Bartel and Shiv Grewal (and was held in conjunction with 'RNAi for Target Validation and as a Therapeutic', organized by Stephen Friend and John Maraganore). The 'MicroRNAs and siRNAs' meeting brought together scientists working on diverse biological aspects of small regulatory RNAs, including microRNAs, small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs and rasiRNAs). Among the themes discussed were the diversity of small regulatory RNAs and their developmental functions, their biogenesis, the identification of their regulatory targets, their mechanisms of action, and their roles in the elaboration of multicellular complexity.

Introduction

MicroRNAs, small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs) are small regulatory RNAs that are unified by their association with Argonaute-family proteins and by their functions as regulatory RNAs that direct the binding of protein complexes to specific nucleic acid sequences. These small RNAs can exert regulation at the transcriptional level, by affecting chromatin structure, or post-transcriptionally, by affecting mRNA stability or translation.

Developmental functions of microRNAs

Animals and plants have hundreds of distinct microRNA genes, whose regulatory roles in development have been implicated by their diverse expression patterns and, in some cases, confirmed by genetic studies in model organisms and in humans (Kloosterman and Plasterk, 2006; Jones-Rhoades et al., 2006). Of the three main classes of small regulatory RNAs discussed at the meeting – microRNAs, siRNAs and piRNAs (Fig. 1) – the developmental roles for microRNAs are the most-clearly evident.

Richard Carthew (Northwestern University, Evanston, IL, USA) described how *miR-7* functions in an intricate network of Notch and epidermal growth factor receptor (EGFR) signaling pathways to specify photoreceptors (Li and Carthew, 2005) and chordotonal sensory organ precursors in *Drosophila*. Carthew proposed that microRNAs such as *miR-7* function in such networks to confer robustness on developmental decisions and to provide genetic buffering under adverse physiological conditions. Indeed, Carthew reported that exposing developing *mir-7* mutant fly eyes to temperature cycles disrupts regular ommatidial patterning. Another example of a microRNA-modulated signaling pathway in *Drosophila* was discussed by Eric Lai (Sloan-Kettering Institute, New York, NY, USA). *miR-315* was identified in a cell-based screen as a potentiator of Wingless (Wg) signaling. Consistent with a role

for *miR-315* in promoting Wg signaling in vivo, among the targets of *miR-315* are the negative regulators of the Wg pathway, Axin and Notum. Moreover, the overexpression of *miR-315* in the presumptive thorax of developing larvae caused dramatic developmental transformations associated with excess Wg activity.

MicroRNAs play important roles in the developmental progression of certain cell lineages and fates. Interestingly, despite the deep phylogenic conservation of some microRNA genes (Pasquinelli et al., 2000), a conserved microRNA can regulate different genetic pathways and developmental processes in different organisms. The genetic analysis of *Drosophila let-7* by Nick Sokol of Victor Ambros's laboratory (Dartmouth Medical School, Hanover, NH, USA) revealed that, unlike the worm *let-7*, which controls developmental transitions mainly in hypodermal cells (Reinhart et al., 2000), the main function of fly *let-7* seems to be to control events in the development of the adult nervous system. In the respective worm and fly *let-7* pathways, the key direct targets of *let-7* also differ.

Phillip Sharp (Massachusetts Institute of Technology, Cambridge, MA, USA) compared the expression profiles of microRNAs and their computationally predicted targets during T cell development, and identified at least one microRNA for each stage whose expression inversely correlated with its predicted-target levels. Of particular note was *miR-181* (also known as *Mirn181a-2* – Mouse Genome Informatics), which had previously been shown to play a role in mouse lymphocyte cell fate specification (Chen et al., 2004), and was found by Sharp to be enriched in double-positive (DP) cells (Neilson et al., 2007). These observations indicate that *miR-181* has a role in the positive selection of DP lymphocytes in response to antigen presentation. Chang-Zheng Chen (Stanford University, Palo Alto, CA, USA) reported how the manipulation of *miR-181* levels in T cells modulates their sensitivity to T cell-receptor signaling, and hence their response to antigens, indicating that *miR-181* may act as a 'rheostat' to tune T cell sensitivity at various stages of T cell development. Chen pointed out that this role of *miR-181* in lymphocyte development exemplifies how microRNAs may be particularly suited to the control of delicately regulated developmental pathways.

Nikolaus Rajewsky (Max-Delbruck-Centrum, Berlin, Germany) presented results of a collaborative project from his and Timothy Bender's laboratory (Harvard Medical School, Boston, MA, USA) showing that, in mouse, *miR-150* (also known as *Mirn150* – Mouse Genome Informatics) plays a role in lymphocyte maturation, possibly via the regulation of the transcription factor cMyb, a direct target of *miR-150*. In transgenic mouse models, moderate ectopic expression of *miR-150* or a graded reduction of cMyb blocks the development of mature lymphocytes, suggesting that lymphocyte maturation is sensitive to the dosage of *miR-150*, which may modulate cMyb levels at crucial times.

Jan Krützfeldt (from Markus Stoffel's group, Institute of Molecular Systems Biology, Zurich, Switzerland) described *mir-375* (also known as *Mirn375* – Mouse Genome Informatics)-knockout mouse phenotypes, in which pancreatic islets display abnormal architecture and a disruption in the balance between alpha and beta cells. Previous overexpression experiments with *miR-375* had indicated that it acts in the regulation of insulin secretion by islet cells (Poy et al., 2004). These new findings suggest an additional role for *miR-375* in pancreatic development. Similar findings were reported by Ronald Plasterk (Hubrecht Laboratory, Utrecht,

[1]Dartmouth Medical School, Hanover, NH 03755, USA. [2]UC Riverside, Riverside, CA 92521, USA.

*Author for correspondence (e-mail: vambros@dartmouth.edu)
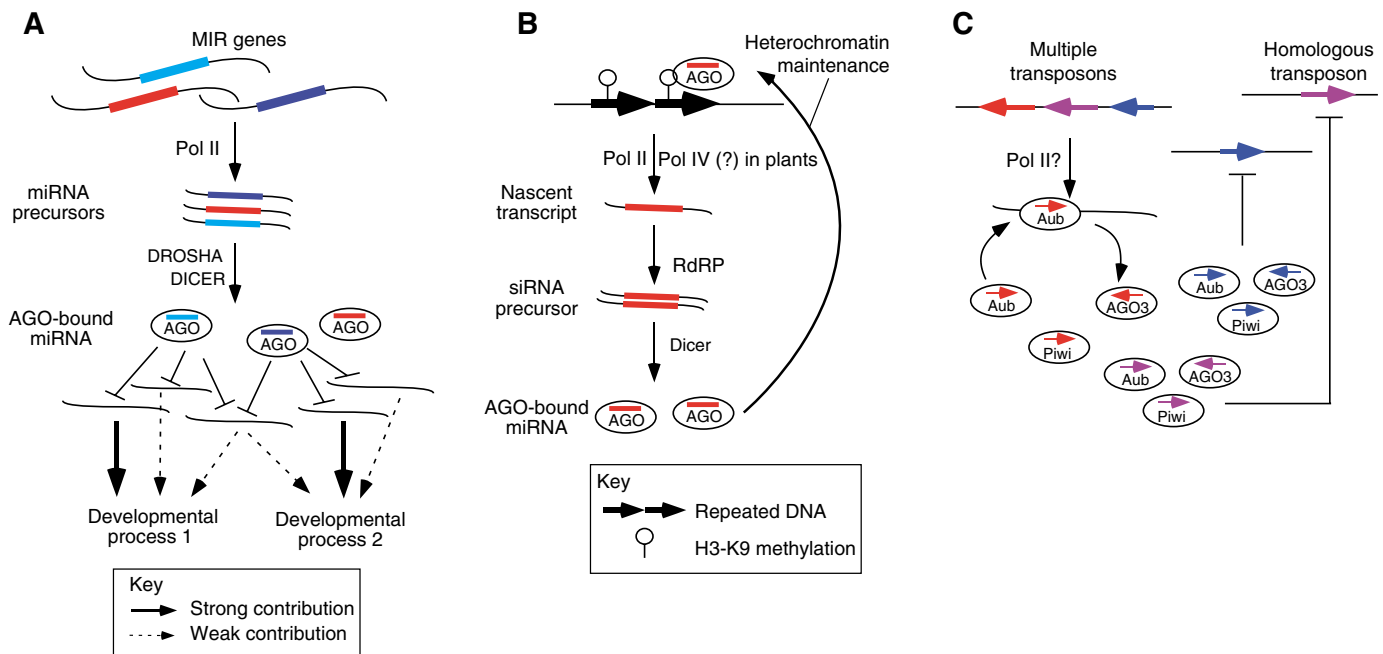
Fig. 1. The biogenesis and function of endogenous small RNAs in animals, and in plants and fungi. (**A**) In animals, microRNA (MIR) genes (top) are transcribed by Pol II into microRNA (miRNA) precursors, which are processed by DROSHA and DICER (DICER1) into miRNAs. The miRNAs are bound by an effector protein Argonaute (AGO) and cause the cleavage or translational inhibition of target mRNAs. In animals, despite the fact that each miRNA is able to regulate scores of target transcripts, genetic studies show that one major target underlies the role of a miRNA in a developmental process. Related miRNAs (light and dark blue) often have shared predicted targets, but genetic studies reveal that members of a miRNA family may have distinct developmental functions. (**B**) Endogenous siRNAs in *S. pombe* and in plants are generated from long transcripts from repeated DNA or transposons via the activities of RNA-dependent RNA polymerases (RdRP) and Dicer. Histone H3 lysine 9 (H3-K9) methylation and the siRNAs complexed with AGO act in a self-reinforcing loop to maintain heterochromatin at the loci. (**C**) Via an unknown mechanism, repeat-associated siRNAs (rasiRNAs) in *Drosophila* are produced from a master locus composed of multiple transposons. These small RNAs bind the Piwi subfamily of Argonaute proteins – Piwi, Aubergine (Aub) and AGO3 – and target homologous transposons scattered around the genome for silencing. An existing rasiRNA guides the cleavage of a target RNA through the slicer activity of the associated Piwi subfamily of Argonaute protein to lead to the production of a second rasiRNA.

Netherlands) for the zebrafish *miR-375*. Knockdown of *miR-375* with morpholino oligonucleotides directed against either the mature microRNA or the stem of the *miR-375* precursor caused similar abnormalities in pancreatic islet organization, strongly supporting the specificity of this phenotype.

Cell type-specific or organ-specific microRNAs are generally considered to be potential determinants of organ or cell type identity (Plasterk, 2006). What role do microRNAs play in pluripotent embryonic stem (ES) cells? Sharp reported the initial characterization of a knockout mouse that lacks *mir-290* through to *mir-295* (*mir-290–295*), an ES cell-specific microRNA cluster (Houbaviy et al., 2003). Homozygous *mir-290–295*^{–/–} animals often die as embryos, although some grow to adulthood. Significantly, female survivors are infertile due to an absence of germ cells, indicating that these microRNAs may function in the maintenance of pluripotent cells in embryos and in the female germ line.

miR-133 and *miR-1* are expressed in mouse mesodermal lineages, and at particularly high levels in muscle. This muscle-specific expression is evolutionarily conserved among animals (Nguyen and Frasch, 2006). Kathryn Ivey (from Deepak Srivastava's group, Gladstone Institutes and University of California, San Francisco, CA, USA) used lentivirus vectors to overexpress *miR-1* and *miR-133* during the in vitro development of embryoid bodies from mouse ES cells, and showed that distinct steps in muscle development are specified by cooperative, followed by opposing, interactions between *miR-1* and *miR-133*. These findings provide insight into

other tissue- or organ-specific microRNAs, where their functional roles may be dynamic, changing and complex. A previously published characterization of *Drosophila mir-1*-knockout mutants (Sokol and Ambros, 2005; Kwon et al., 2005) suggested that *miR-1* maintains differentiated muscle during larval growth. In mice, knockout of one of the two *mir-1* loci (*mir-1-2*, also known as *Mirn1-2*), as reported by Srivastava's laboratory, leads to heart defects, including apparent hyperplasia of myocytes. So, although the *mir-1* sequence and its muscle-specific expression is essentially identical between insects and mammals, it is not yet clear whether *miR-1* function is entirely conserved. Anindya Dutta (University of Virginia, Charlottesville, VA, USA) reported a function for *miR-206* in the myogenic differentiation of mouse C2C12 cells – a model for regenerative myogenesis – in culture (Buckingham, 2006). When C2C12 cells are removed from serum, they produce differentiated muscle and show *miR-1, miR-133* and *miR-206* upregulation. *miR-206* appears to be required for muscle differentiation in response to serum starvation.

microRNAs and cancer

Carlo Croce (Ohio State University College of Medicine, Columbus, OH, USA) discussed the importance of reliable diagnostic and prognostic markers for cancer, and how microRNA expression patterns appear to be more reliable than mRNA expression patterns for distinguishing the tissues of origin of human tumors (Lu et al., 2005). Croce reported that both *MIR-15* and *MIR-16* are tumor

suppressors in chronic lymphocytic leukemia (CLL), and that they target B-cell CLL/lymphoma 2 (*BCL2*). microRNA expression patterns distinguish an aggressive from an indolent form of CLL, potentially permitting earlier intervention with appropriate therapy. Similarly, measurements of *MIR-155* and *LET-7* levels distinguish Stage-1 human lung cancers of poor prognosis from those with a better prognosis far sooner than alternate assays.

Anna Krichevsky (Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA), in collaboration with Khalid Shah (Massachusetts General Hospital, Boston, MA, USA), revealed a role for *MIR-21* (also known as *MIRN21* – Human Gene Nomenclature Database) in mammalian brain tumors. The pre-treatment of human brain tumor cells with anti-*MIR-21* oligonucleotides inhibits their tumorigenicity when transplanted into mice. *MIR-21* overexpression is a hallmark of certain classes of brain tumors, so these results suggest that *MIR-21* could indeed contribute to the malignancy of these tumor cells, probably via the repression of one or more tumor suppressor genes.

The converse situation, where tumor-suppressive microRNAs may function to repress oncogenes, was illustrated by David Bartel (Whitehead Institute and MIT, Cambridge, MA, USA). The oncogenic behavior of certain mutated forms of High mobility group A2 (HMGA2) in humans is induced by the deletion of the *HMGA2* 3′ UTR (Mayr et al., 2007), which contains several evolutionarily conserved sites for *LET-7*, a microRNA with potential tumor-suppressive activity (Johnson et al., 2005).

Finding and validating microRNA targets in animals

Current strategies to identify the regulatory targets of microRNAs include the computational prediction of base-pair matches (either near-perfect, as in plants, or partial base-pairing, as is more common in animals), followed by experimental validation. A central principle of animal microRNA target identification is the occurrence of complementary sequences in 3′ untranslated regions (UTRs), and matches to the 'seed' region (nucleotides 1-8, from the 5′ end) of the microRNA. These relatively lax criteria can result in numerous targets being predicted for a given microRNA. However, many predicted interactions are evolutionarily conserved, indicating that many animal genes are under selective pressure to maintain microRNA target sites.

Interestingly, as Carthew discussed, experimental evidence suggests a greater functional specificity for microRNA-targeting than is often computationally predicted. Ambros elaborated this point in reviewing the role of *let-7* and *lin-4* in worm developmental timing. Although *lin-4* and *let-7* are each predicted to have many conserved targets (Lall et al., 2006), the pleiotropic phenotypes that result from the loss of either microRNA can be suppressed by the mutation of just one particular target gene (Ambros, 1989; Slack et al., 2000). Ambros reported that this also applies to *let-7* function in *Drosophila*, where the severe behavioral defects of the *let-7* deletion mutant are efficiently suppressed by the mutation of a single predicted target, the *abrupt* transcription factor. These results suggest that the effects of microRNAs on gene networks may not be immediately apparent from computational predictions alone, and that genetic analysis may be the most definitive way to determine the relative phenotypic impact of specific predicted interactions.

Nevertheless, computational methods can efficiently identify candidate targets, and improvements in these methodologies were presented by Chris Burge (Massachusetts Institute of Technology, Cambridge, MA, USA) and Bartel. From separate studies, a refined approach to seed-match target prediction was presented. Both

groups analyzed data from experiments in which microRNA levels were perturbed in various ways. The resultant changes in target mRNA levels were monitored by microarray hybridization analysis. The strength of a microRNA-target interaction, as revealed by the level of target mRNA knockdown, reveals how features of the target site (of a particular microRNA) can influence these interactions. Bartel reported that favorable target-site features include the length and position within the microRNA of the seed match, A/U-rich sequences immediately surrounding the seed match, and the distance of the site from the middle of the UTR. Curiously, Burge found that an A or U at position 9 of the target site was an efficient predictor of an effective interaction, regardless of the base-pairing at that position. Burge also reported that endogenous vertebrate microRNA-targeting follows similar or identical rules to that of exogenous microRNAs. Both Burge and Bartel found that conserved seed matches tend to provide more efficient knockdown than non-conserved seed matches, perhaps in part due to the evolutionarily conserved favorable features, such as those reported above. They also found that different types of seed matches with specific patterns of complementarity to mRNAs consistently mediate different levels of mRNA downregulation.

Ultimately, assaying only the changes in target mRNAs levels (without also assaying the corresponding proteins) is not sufficient to characterize all functional targets. Thus, a comprehensive picture of what constitutes an effective microRNA-mRNA interaction requires protein-level assays and the verification of functional microRNA-target complex formation in vivo. A promising proteomics-based validation method was reported by Plasterk. Wild-type or microRNA-mutant worms were labeled with heavy or light isotopes, and protein fractions from these populations were mixed, separated by 2D gel electrophoresis and analyzed by mass spectrometry. In this differential approach, microRNA target proteins are expected to be upregulated in the mutant. In principle, this method should permit the direct measurement of protein level changes, independently of whether the corresponding mRNA is downregulated. This method may be a particularly powerful tool for homogeneous samples, such as cultured cells, or sorted cell types from complex tissues. Thomas Tuschl (Rockefeller University, New York, NY, USA) reported the co-immunoprecipitation of Argonaute protein along with associated mRNAs from cultured human cells. In conjunction with proteomics-based assays, the biochemical identification of specific microRNA-target complexes should permit the direct validation of functional endogenous microRNA-target interactions.

microRNA-mediated inhibition of target gene expression

microRNAs exert two general effects on target mRNAs: translational repression and a reduction in mRNA level (Carthew, 2006; Pillai et al., 2006). Both of these effects are post-transcriptional, and they involve different mechanisms. A few animal microRNAs have sufficient complementarity to the mRNA, which allows the mRNA to be sliced between 10 and 11 nucleotides from the 5′ end of the microRNA, as is seen in traditional siRNA-mediated RNA silencing (Yekta et al., 2004). However, most animal microRNAs imprecisely match their targets and cause target mRNA destabilization by other (non-slicer) mechanisms, such as de-adenylation and decapping (Wu et al., 2006; Giraldez et al., 2006), and/or some form of translational repression (Olsen and Ambros 1999). Most plant microRNAs have near-precise matches to their targets and lead to mRNA cleavage (Tang et al., 2003; Jones-Rhoades et al., 2006). However, translational repression can occur

for certain microRNA-target interactions in plants (Aukerman and Sakai 2003; Chen 2004; Arteaga-Vazquez et al., 2007). Olivier Voinnet (Institut de Biologie Moléculaire des Plantes, Strasbourg, France) suggested that translational regulation in plants may be common despite the prevalence of nearly perfect matches. His group performed mutagenesis of an *Arabidopsis* line bearing a green fluorescent protein (GFP) transgene under the control of *mir171*. One class of mutants isolated showed elevated levels of GFP protein but not of *GFP* mRNA, as well as elevated levels of endogenous proteins, but not of mRNAs predicted to be targeted by other microRNAs. The presence of this class of mutants suggests that plant microRNAs can lead to both transcript cleavage and translational inhibition of target mRNAs.

Sharp discussed how the structural and base-sequence features of an animal microRNA-target hybrid affect whether or not the microRNA causes RNA degradation or translational inhibition. Using microRNAs transfected into animal cells containing a set of mutant reporter constructs, Sharp tested alternative base-pairings and mismatches at positions 9, 10 and 11 of a microRNA that otherwise matched at all other positions. Certain structures were found to cause potent translational repression without affecting mRNA level, whereas others led to mRNA reduction without translational repression. These results suggest that the outcome of a microRNA-target interaction may depend on structural features of the microRNA-target duplex that are recognized by microRNA-associated proteins.

Can specific microRNAs or associated 3′ UTRs have context-dependent effects on knockdown? Elisa Izaurralde (Max Planck Institute, Tuebingen, Germany) used natural 3′ UTRs fused to a luciferase reporter to perform genome-wide RNA interference (RNAi) screens for proteins required for microRNA function, and isolated components of P-bodies, and de-adenylation and de-capping enzymes (Behm-Ansmant et al., 2006). Significantly, only certain targets were repressed by a microRNA in the absence of de-adenylation or decapping enzymes, which indicates that decapping (and subsequent degradation) of de-adenylated mRNAs is the primary mode of repression by some, but not all, microRNA-target interactions.

Progress was reported in the search for potential mechanisms of translational repression by microRNAs. Ramin Shiekhattar (Centre de Regulació Genòmica, Barcelona, Spain) reported that the eukaryotic translation initiation factor 6 (eIF6; also known as Itgb4bp – Mouse Genome Informatics), along with components of the 60S ribosomal subunit, can be co-immunoprecipitated with Argonaute 2 (Ago2; also known as Eif2c2 – Mouse Genome Informatics) from animal cells. Moreover, RNAi knockdown of *eIF6* in animal cells, as well as in *C. elegans*, results in the measurable de-repression of microRNA target expression. *eIF6*, tethered to an mRNA via the Ago2-microRNA complex, could bind the 60S ribosomal subunit, preventing the formation or stability of 80S complexes, and repressing translational initiation and/or elongation. Marianthi Kiriakidou (from the Mourelatos group, University of Pennsylvania, Philadelphia, PA, USA) drew attention to amino acid similarities between a region of the Human AGO2 protein and the 7mG cap-binding domain of EIF4E. Indeed, AGO2 displayed 7mG cap-binding activity, and the mutation of phenylalanine residues key to cap-binding activity impaired the translational repressive activity of AGO2 in a tethering assay.

microRNA activity can be modulated by additional protein co-factors. One such example, as discussed by Witold Filipowicz (Friedrich Meisner Institute, Basel, Switzerland), involves the human HuR (also known as ELAVL1 – Human Gene Nomenclature Database) protein, which is exported from the nucleus of liver cells in response to stress. This Elav-family RNA-binding protein binds to an element in the 3′ UTR of *CAT-1* (also known as *SLC7A1* – Human Gene Nomenclature Database) mRNA and de-represses CAT-1 protein production by interfering with the repressive activity of *miR-122* (*MIRN122A*) (Bhattacharyya et al., 2006). *miR-122* activity leads to the sequestration of *CAT-1* mRNA in P bodies, whereas, upon HuR binding, *CAT-1* mRNA exits the P bodies and is translated, perhaps via the displacement of the microRNA from the mRNA. Similarly, Antonio Giraldez (Yale School of Medicine, New Haven, CT, USA) reported how the *Nanos* 3′ UTR mediates the repression of a *mir-430* family microRNA sensor in zebrafish somatic cells, but not in the germline. The effect is due to a property of the 3′ UTR outside of the *miR-430* sites and is consistent with the presence of a germ-cell-specific factor that blocks *miR-430* activity in the context of some 3′ UTRs, but not others (Mishima et al., 2006).

One area that is beginning to garner more attention is the intrinsic secondary structure of the mRNA in the vicinity of the microRNA target site, and how this could affect the accessibility of the microRNA to its complementary sequences (Robins and Padgett, 2005; Zhao et al., 2005). Bartel commented that two features of functional sites – A/U-rich sequences surrounding the site and preferential placement of the more-effective sites at the ends of the 3′ UTR – could influence the accessibility of the microRNA-binding site in the 3′ UTR. Renée Schroeder (Max Perutz Laboratories, Vienna, Austria) reported results from RNA-induced silencing complex (RISC)-binding and target-cleavage experiments performed in collaboration with Javier Martinez (IMBA, Vienna, Austria). By careful quantitation of the velocity of the RISC-cleavage reaction, and the biochemical verification of predicted target secondary structures, a strong effect of target accessibility on RISC binding was convincingly demonstrated. It will be exciting to see if these biochemical analyses can be adapted to the non-cleaving activity of microRNA-guided RISC, and whether the influence of a target secondary structure can also be observed for natural 3′ UTR substrates.

Gene regulation by piRNAs and endogenous siRNAs

Recent advances have illuminated the surprisingly diverse modes of small RNA-guided gene regulation by Argonaute-based mechanisms (Fig. 1). The Piwi class of Argonaute proteins is essential for spermatogenesis in mice and for gametogenesis in flies (Cox et al., 1998; Kim, 2006); in the past year or so, a new class of small RNAs – piRNAs – have been found to be associated with Piwi family proteins (Saito et al., 2006; Vagin et al., 2006; Grivna et al., 2006; Aravin et al., 2006; Girard et al., 2006; Lau et al., 2006). Although piRNA function in gametogenesis is still unclear, Gregory Hannon (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA) and Mikiko Siomi (University of Tokushima, Tokushima, Japan) reported progress in understanding the biogenesis and activity of *Drosophila* repeat-associated siRNA (rasiRNAs), which are similar to mammalian piRNAs in many respects.

Hannon presented results from studies on the biogenesis and function of a cluster of rasiRNAs from the *flamenco* locus, a transposon-rich heterochromatic locus on the fly X chromosome that serves as a master control locus to silence *gypsy*, *ZAM* and other transposons scattered around the genome. The *flamenco* locus spawns rasiRNAs that presumably interact with, and silence, homologous transposon sequences throughout the genome. Features of these rasiRNAs reveal aspects of their biogenesis via a 'ping-pong' mechanism that occurs without the involvement of Dicer

activity and results in the amplification of rasiRNAs. These results suggest that, one way to generate rasiRNAs is by Slicer-mediated cleavage of target transcripts.

Siomi presented similar findings for the *Suppressor of stellate* locus, which generates rasiRNAs that act in an Aubergine-dependent manner to silence *Stellate* (also known as *SteXh* – FlyBase) mRNA/protein in fly testes. Sarah Elgin (Washington University, St Louis, MO, USA) showed that *Drosophila* Piwi and Aubergine are required for repeat-induced gene silencing. In a collaborative study with Haifan Lin (Yale University, New Haven, CN, USA), Piwi was found to interact with Heterochromatin protein 1 (HP1) in yeast two-hybrid assays, which implicates Piwi, and perhaps piRNAs, in heterochromatin formation.

Histone modification is crucial to the regulation and stabilization of gene expression, and small RNAs play a central role in this process. Shiv Grewal [National Institutes of Health (NIH), Bethesda, MD, USA] provided evidence for the existence of a self-reinforcing loop mechanism, in which heterochromatin components stably tether RNAi machinery across chromosomal domains that contain certain repeats. These heterochromatin-bound RNAi factors may selectively produce repeat siRNAs in order to fend off future invasion by similar sequences (Grewal and Jia, 2007). Grewal introduced a multi-enzyme silencing effector complex in *Schizosaccharomyces pombe* (termed SHREC) that connects the RNAi pathway to chromatin remodeling. SHREC binds the heterochromatin platform established by the RNAi machinery and effects transcriptional gene silencing via the activities of the histone deacetylase Clr3 and a SNF2 family ATPase Mit1 (Sugiyama et al., 2007). Martin Gorovsky (University of Rochester, Rochester, NY, USA) illustrated the importance of a class of small RNAs (scanRNAs) for proper conjugative reproduction in the protist *T. thermophila*. scanRNAs direct histone modifications in the newly replicated 'somatic' nucleus.

Plants also use similar mechanisms to maintain heterochromatin, as discussed by David Baulcombe (Sainsbury Laboratory, Norwich, UK) and Marjori Matzke (Gregor Mendel Institute, Vienna, Austria) (Fig. 1). Matzke reported the identification of loci that are methylated and silenced by endogenous siRNAs in an RNA polymerase (Pol) IVb-dependent manner. The Pol IVb-silenced loci include repeats, long terminal repeats (LTRs) of retrotransposons, and DNA transposons. Many of the Pol IVb-silenced loci also contain short internal tandem repeats, which are thought to help sustain RNA-dependent RNA polymerase (RdRP) activity in generating double-stranded RNA (dsRNA), from which, siRNAs are generated through Dicer activity.

Steven Jacobsen (University of California, Los Angeles, LA, USA) presented genome-wide profiles of DNA methylation (Zhang et al., 2006) and histone H3 lysine 27 (H3K27) trimethylation (me3) in *Arabidopsis*. H3K27me3 marks in animals span large regions of developmentally important loci, such as the Hox clusters and certain gene promoters, and are regulated by three Polycomb repressor complexes; Polycomb repressive complex 1 (PRC1), PRC2, and Pho-repressive complex (PhoRC). Plants, however, only possess the PRC2 complex, and this might explain why H3K27me3 tends to be restricted to small regions (within 1 kb) in plants. H3K27me3 marks are found in the main body (not at the promoter or 3′ end) of genes on euchromatic chromosomal arms. H3K27me3 genes tend to be expressed in a tissue-specific manner and probably have developmental roles. Curiously, the H3K27me3 marks do not correlate with the presence of small RNAs, nor with DNA methylation.

Worms also carry out ongoing silencing of endogenous genes by RNAi (Lee et al., 2006). Endogenous worm siRNAs are probably generated by mechanisms similar to those that produce secondary siRNAs, which induce long-lasting, heritable silencing (Sijen et al., 2007; Pak and Fire, 2007). Andrew Fire's (Stanford University, Palo Alto, CA, USA) results indicate that the majority of secondary siRNAs are produced by de novo synthesis of short RNAs by RdRp. Craig Mello (University of Massachusetts, Worchester, MA, USA) reported that endogenous siRNAs are also produced de novo by RdRp and that they depend on the activity of Dicer related helicase (DRH-3). Mello suggested that much of the worm genome is subject to ongoing, epigenetic and heritable gene silencing, and, therefore, natural selection could maintain favorable epigenetically determined variations in gene expression.

Evolution of small regulatory RNA function
Several plant and animal microRNAs are highly conserved within their respective kingdoms (Pasquinelli et al., 2000; Axtell and Bartel, 2005), indicating that microRNA-based gene regulation emerged early during the evolution of both plants and animals, perhaps in conjunction with the acquisition of complex multicellular forms. The phylogenetic distribution of conserved microRNAs in animals (Sempere et al., 2006; Prochnik et al., 2007) indicates that at least 27 of the conserved microRNA families arose in the ancestor of all bilaterians (the 'urbilaterian'), whereas perhaps just three conserved families evolved earlier, in an ancestor to bilaterians and cnidarians. The rapid expansion of microRNA number and type in an urbilaterian ancestor suggests that these post-transcriptional regulators played a role in the adaptive diversification of pre-existing transcriptional and cell-cell signaling pathways required for the evolution of complex and diverse organs and body plans.

Many microRNAs and their target interactions appear to be rapidly evolving, suggesting an ongoing potential for microRNAs to drive animal and plant diversity. James Carrington (Oregon State University, Corvallis, OR, USA) revealed how his laboratory recently identified non-conserved microRNA loci in *Arabidopsis*, supporting the view that new plant miRNA loci may evolve from the inverted duplication of founder loci, producing a hairpin RNA. Dicer-like 4 (DCL4)-processing of the new hairpin leads to the generation of siRNAs, and microRNAs would subsequently evolve via DCL1 activity as the hairpin RNA accumulates mismatches. Carrington noted that, in plants, the recently evolved microRNAs seem to be far less likely to target transcription factors than conserved microRNAs (Fahlgren et al., 2007); indeed, some do not have any predicted targets, suggesting that they could be on an evolutionary path to elimination. Carthew found that *Drosophila* species-specific microRNAs are expressed at relatively low levels and, hence, some of these microRNAs could be evolutionarily neutral. Rajewsky pointed out that some of the non-conserved targets of conserved microRNAs could be similarly neutral. These exciting ideas suggest that organisms contain a vast reservoir of potentially important microRNA-target regulatory interactions poised for natural selection.

Phil Zamore (University of Massachusetts, Worchester, MA, USA) and Narry Kim (Seoul National University, Seoul, Korea) described aspects of microRNA biogenesis that profoundly affect microRNA expression and activity, and hence are crucial factors in microRNA function and evolution. Zamore reported structural features of microRNA precursors in flies that govern the sorting of a microRNA to the correct Argonaute effector complex. Kim noted that most mammalian microRNAs are encoded in the sense strand of introns, often within protein-coding genes, and reported on

studies of the mechanistic and regulatory relationship between pre-mRNA splicing of the host gene and DROSHA (also known as RNASEN – Human Gene Nomenclature Database)-mediated processing of the intronic microRNA.

Are endogenous siRNAs broadly conserved in animals? The comparative genomics of RNAi pathways and proteins between plants and animals indicate that many endogenous siRNAs are products of RdRPs; interestingly, among animals, only nematodes are reported to contain such genes (Cerutti and Casas-Mollano, 2006). The known features of mammalian piRNAs and *Drosophila* rasiRNAs from the *flamenco* locus do not support the possible existence of a precursor dsRNA or an RdRP-based mechanism. Even the deep-sequencing of small RNAs from mouse ES cells (reported by Sharp) did not reveal endogenous siRNAs. However, Baulcombe issued a cautionary tale of the unicellular algae *Chlamydomonas reinhardtii*, which lacks an RdRP gene in its genome, but expresses a class of endogenous siRNAs that bears the hallmarks of endogenous siRNA clusters that are seen in plants and worms.

Conclusion

The field of small regulatory RNAs is enjoying rapid progress. We continue to be surprised by the diversity of small RNAs and their regulatory mechanisms, and it is likely that the complexity of these phenomena will continue to blossom in the coming months and years. Some of the immediate challenges are to elucidate the developmental roles of piRNAs, and the mechanisms of biogenesis of piRNAs, rasiRNAs and endogenous siRNAs. Precisely how do small RNAs mediate the epigenetic regulation of gene expression? By what mechanisms do microRNAs influence the translational efficiency and stability of target mRNAs? What factors, specific or non-specific, modulate microRNA target recognition and repression? Can microRNAs activate the expression of a target? One challenge for the future will be to understand the evolutionary mechanisms that have fixed the entire 21 nucleotide sequence of certain microRNAs for hundreds of millions of years (Pasquinelli et al., 2000; Sempere et al., 2006; Prochnik et al., 2007). The answers to these and other questions may emerge when we return to the Keystone Symposium next year.

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