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Introduction to Gene Regulation by Small RNAs

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Gene regulation by small RNAs is now recognised to be critical in animal development and physiology. Human disease conditions including cancer and cardiovascular disease are often associated with the misregulation of microRNAs. MicroRNAs (miRNAs) are ~22 nucleotide non-coding RNAs that are generated by the activity of the RNase III enzyme, Dicer. The biologically relevant target messenger RNAs (mRNAs) for the majority of microRNAs remain unknown. Target mRNAs are controlled by microRNAs at the step of translation initiation or elongation. Piwi-interacting RNAs (piRNAs) are ~24–30 nucleotide non-coding RNAs that are generated by a Dicer-independent mechanism and function in the germline to prevent the spreading of mobile genetic elements.

1.1 Introduction

Once considered the sole province of proteins, control of gene expression by a small RNA was first described in 1993 when Victor Ambros and

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co-workers identified the ~22 nucleotide small RNA encoded by the *lin-4* gene in *C. elegans*.¹ This early research defined many of the key elements of post-transcriptional regulation of gene expression by microRNAs. From genetic and molecular evidence, it was clear that *lin-4* represses *lin-14* activity. Therefore, when *lin-4* was found to have antisense complementarity to sequence motifs in the *lin-14* 3' untranslated region (UTR),² the model of a miRNA repressing target gene activity through interaction with the 3' UTR of a target mRNA was established. However, because no homologues of *lin-4* were identified in higher organisms, it was unclear how much of this regulatory mechanism was conserved. The *lin-4* small RNA remained an oddity until 2000, when the second gene encoding a small regulatory RNA, *let-7*, was identified, first in worms³ and subsequently in a wide array of species including flies, fish, mice and humans.⁴ There now exist several classes of endogenous small RNA regulators of gene expression, including miRNAs as well as piwi-associated small RNAs (piRNAs). Thus, *lin-4* is now viewed as an 'emissary' for a newly-appreciated class of regulatory molecules⁵ rather than the 'deviant' it may have once been considered.

1.2 Gene Regulation by MicroRNAs

A miRNA is defined as a small, single-stranded RNA, 19–24 nucleotides in length that is generated following the cleavage of a longer precursor transcript by the RNase III-type enzyme Dicer. Loci encoding miRNAs are found in the genome in independent transcription units, in clusters of multiple miRNAs, or within introns of annotated genes.⁶ Experimentally, miRNAs have been identified by sequencing complementary DNA (cDNA) libraries derived from size-selected RNA samples or by computational predictions. These candidate miRNAs are then validated by using detection methods such as northern blotting, RT-PCR, or microarray analysis.⁷ These methods have led to the identification of thousands of miRNAs in animals, plants and viruses. Release 15 of the miRBase database lists 14,197 entries for miRNAs. The identification of miRNAs has thus far vastly outpaced miRNA functional analysis.

These miRNAs are generated in a cell following multiple processing steps in both the nucleus and the cytoplasm to generate the ~22 nt mature

miRNA.⁸ Unlike other small RNAs such as transfer RNAs or U6 small nuclear RNA, most miRNAs are transcribed by RNA Polymerase II (Pol II) as long RNA precursors called primary miRNAs (pri-miRNA). Like other Pol II transcripts, pri-miRNAs are capped and polyadenylated.^{9,10} Pri-miRNAs undergo co-transcriptional processing mediated by the Microprocessor complex, which includes the RNase III enzyme Droscha and DGCR8/Pasha, in order to generate a ~70 nucleotide stem-loop precursor miRNA (pre-miRNA).^{11,12} Those miRNAs that reside in the introns of annotated genes can be processed either by the Droscha complex before the splicing reaction or by the pre-mRNA splicing machinery to generate the pre-miRNA.^{13–15} This ~70 nt stem-loop pre-miRNA is exported from the nucleus into the cytoplasm where it is processed by another RNase III enzyme, Dicer.^{16–20} The mature, single-stranded miRNA functions in a protein complex called a miRISC (RNA-induced silencing complex), to direct the repression of target mRNAs.

1.2.1 Mechanisms for microRNA-mediated regulation of gene expression

The function of a miRNA contained in a miRISC is to identify target mRNAs through sequence complementarity in order to block protein production. An Argonaute protein is a core constituent of the miRISC and is necessary for miRNA activity. Flies that lack activity of the Argonaute-encoding gene, *dAGO1*, die during embryogenesis or early larval development and display defects in miRNA maturation or stability.^{21,22} Similarly, worms that lack activity of two Argonaute-encoding genes, *alg-1* and *alg-2*, have reduced miRNA levels and die early in development.¹⁷ Although worms have at least 26 genes encoding Argonaute proteins, *alg-1* and *alg-2* are the only ones that have been found to function in the miRNA pathway.^{17,23} Argonaute proteins contain a PAZ domain that can recognise miRNAs by their characteristic Dicer-generated 3' overhangs as well as a PIWI domain that is important for target repression by miRNAs.^{24,25} Nearly all miRNAs in animals bind to their mRNA targets with imperfect complementarity. This imperfect complementarity in binding results in bulges present in the miRNA–mRNA duplex; this precludes the specific Argonaute-mediated cleavage known as Slicer activity and instead leads to

target mRNA repression of translation. In contrast, in the RNA interference (RNAi) pathway, Slicer activity cleaves mRNA targeted by a perfectly matched small interfering RNA (siRNA).

While it is clear that nearly all miRNA targets are not specifically cleaved by Slicer activity, the exact mechanism for post-transcriptional repression is not yet fully understood and remains controversial.²⁶ It may be that multiple mechanisms for miRNA-mediated repression exist and the fates of individual target mRNAs will vary. Early studies demonstrated that the *lin-4* miRNA directed the dramatic reduction of LIN-14 and LIN-28 protein levels while effecting little change on their mRNA levels. Target mRNAs co-fractionate with polyribosomes along with miRNAs.^{27,28} These polysomes are sensitive to puromycin indicating that they are in the process of active elongation.^{29–31} This result suggested that miRNA-mediated repression can occur after the initiation of translation. Inhibition of translation can occur post-initiation due to a block in translation elongation or due to degradation of the nascent polypeptide chain.^{30,31}

In addition to blocking translation at a step post-initiation, miRNAs can direct the degradation of the target mRNA, due to decapping or deadenylation. For example, miR-430 is expressed in zebrafish during early embryogenesis and mediates the deadenylation and loss of maternal mRNA targets at the maternal-to-zygotic transition.³² Deadenylation and degradation of target mRNAs is also seen in flies and in mammalian cells.^{33,34} One model is that miRNAs function to direct target mRNAs towards processing bodies, or P-bodies, that contain enzymes involved in RNA decay for storage or for degradation.^{35,36}

However, more recent studies suggest that miRNA-mediated repression of target mRNAs can also occur at the step of translation initiation. First, in cultured mammalian cells, a target mRNA reporter shifts towards the lighter fractions of a sucrose gradient when repressed by a miRNA, suggesting a block of translation initiation.³⁷ In contrast, as discussed above, *lin-14* and *lin-4* are found in the heavier, polyribosome-containing fractions, suggesting a block after initiation. Secondly, reporter mRNAs under the regulation of viral internal ribosome entry sites (IRES) do not appear to be repressed by miRNAs, suggesting that recognition of the 5' cap of a target mRNA may be required for miRNA-mediated repression.^{37,38} Thirdly, human Ago2 is able to associate with the m⁷G structure and this

association is necessary for translational repression.³⁹ Lastly, in order to determine the mechanism for repression, proteins that function in the miRISC have been identified. Factors of the core translational initiation complex such as polyadenylate-binding proteins, an eIF4G orthologue and a component of the 40S ribosome have been demonstrated to associate with the miRISC factors, AIN-1 and AIN-2.⁴⁰ Notably, proteins in the decapping complex were not identified.⁴⁰ Thus, one model for miRISC activity is that proteins of the miRISC bind to the cap of target mRNAs and block access of key initiation factors to the target mRNA.

These models are not mutually exclusive, as there is evidence to support multiple mechanisms for miRNA-mediated repression of target mRNAs. One possibility is that miRNAs may effect these different sets of changes on their targets dependent upon other regulatory proteins associated with a miRISC or other modifications to the miRISC or to the target mRNA. In support of this, it was observed that the promoter region of a target could govern the type of miRNA-mediated regulation. Those mRNAs under the regulation of an SV40 promoter are repressed at the initiation step whereas those mRNAs under the regulation of a TK promoter are repressed after the initiation step, as determined by sucrose gradient analysis.⁴¹ Factors that associate with the target mRNAs in order to determine the mechanism by which a target is repressed have not been identified.

1.2.2 Identification of microRNA-regulated target mRNAs

In animals, miRNAs bind to their target mRNAs with imperfect sequence complementarity. As stated above, functionally, this imperfect binding precludes Slicer-mediated mRNA cleavage of target mRNAs. Computationally, this imperfect binding precludes direct identification of target mRNAs by simple analysis of the nucleotide sequence of 3' UTRs. There have been various strategies employed to identify candidate target mRNAs for individual miRNAs in many species. One strategy is to identify 3' UTRs that have binding sites with high complementarity to the 7–8 nucleotide 'seed' sequence at the 5' end of miRNAs and that are conserved across closely related species. Such approaches have identified thousands of possible miRNA target mRNAs in humans, flies, worms and other

animals.^{42–46} In addition, surrounding sequence motifs around binding sites,⁴⁷ compensatory binding of the 3' end of the miRNA,⁴² combinatorial control of a mRNA by multiple miRNAs⁴⁴ and secondary structure of the 3' UTRs of targets^{48,49} have been incorporated into target prediction algorithms and may improve the accuracy of predictions.

However, while these predictions are highly useful tools to begin to understand the possible functions of specific miRNAs, it is expected that these lists contain many false positives and may fail to identify biologically relevant targets that do not fit the current models of miRNA–mRNA binding. In addition to computational approaches, biochemical approaches have been taken to identify biologically relevant miRNA targets.^{40,50–53} Combining these bioinformatic and biochemical data sets⁵⁴ is expected to greatly strengthen the ability to accurately predict target mRNAs for individual miRNAs.

The biological significance of miRNA-mediated gene regulation has been characterised by analysis of mRNA and protein levels following changes in miRNA activity. Transfection of a single miRNA into cultured cells results in modest changes in mRNAs levels, many of which are predicted to be direct targets of the transfected miRNA.⁵⁵ In addition to analysis of changes at the mRNA level, analysis of changes at the protein level can also be used to determine the function of specific miRNAs. Recent advances in proteomics approaches were used to determine the effect of introducing a single miRNA to cultured cells or inhibiting the activity of an endogenous miRNA.^{56,57} Two important themes emerge from these studies: first, the impact of miRNA regulation on an individual gene's activity is modest, with most changes at the two-fold level, and second, hundreds of genes displayed change in protein expression. Therefore, as these cases of mis-expressed miRNA activity suggest, some miRNAs function to modulate the overall protein production in a cell rather than strongly repress a specific subset of targets.

An important function for some miRNAs is to fine-tune the activity of a suite of target mRNAs, acting as 'micromanagers' of gene expression.⁵⁸ The ability of a single miRNA to bind a target mRNA with only seven nucleotides of complementarity can allow for the repression of a large number of genes, which may globally influence the protein production of a particular cell. For example, introduction of the brain-specific miRNA,

miR-124, into HeLa cells results in changes in mRNA levels of 174 genes marking a global shift in the expression profile of HeLa cells to more closely resemble the profile of brain tissue.⁵⁵ Thus, miR-124 may act to promote or stabilise tissue-specific identity in the brain through modest repression of a suite of targets. In addition, miR-430 and miR-309-cluster miRNAs in fish and flies, respectively, regulate a suite of target mRNAs at the maternal-zygotic transition in order to remove hundreds of maternal transcripts from the early embryo.^{32,59}

In contrast to examples of individual miRNAs that regulate a large set of targets, other miRNAs appear to fine-tune, or modulate, the expression of only a few key mRNA targets. These miRNAs can function to subtly repress targets in order to keep target protein levels within an optimal range. In these cases, it is expected that miRNA and target are co-expressed and also that both too little and too much target protein would cause abnormalities. One example of this type of miRNA-target relationship is miR-9a regulation of *senseless* activity during sensory bristle development in flies.⁶⁰ In the absence of miR-9a, there is a small and variable increase in the number of sensory bristles. Mir-9a regulation may function to keep *senseless* activity below a threshold in those cells not fated to become sensory bristles. While transcriptional control can be sufficient, the additional post-transcriptional control provided by miR-9a confers robustness on this developmental pathway and ensures that no ectopic bristles are specified.

While some miRNAs that are co-expressed with target mRNAs may act to modulate protein levels or act in conjunction with transcriptional control of targets, other miRNAs function to fully repress the activity of their targets. Only a few of these so-called 'switch' targets have been identified to date. These would be the most readily identified by genetic screens. Indeed, the first miRNAs identified, *lin-4* and *let-7*, were found using forward genetics and act to nearly completely repress the target mRNAs, *lin-14* and *lin-41*, respectively.^{61,62} Mutations in these genes can suppress the strong, penetrant phenotypes associated with loss of *lin-4* or *let-7*. In flies, the *let-7* miRNA regulation of neuromuscular junction development is possibly mediated through the control of the *abrupt* gene.^{63,64} Thus, in several cases the functions of miRNAs are mediated primarily through strong repression of single targets or a small number of

targets. However, although there are a large number of miRNA-encoding genes that have been identified in flies, fish and worms, only a tiny fraction of these have been found using forward genetic screens. Although this could partly be due to the relatively small target size of miRNA-encoding loci, it suggests that ‘switch’ targets that are essential for developmental pathways are rare. Whether they are fine-tuning or switch targets, the identification of the biologically relevant targets controlled by specific miRNAs to regulate animal development or physiology is a crucial challenge in the field.

1.3 Gene Regulation by piRNAs

In 2006, a new class of RNA regulatory molecules of about ~26–32 nucleotides in length, the Piwi-interacting RNAs (piRNAs), was identified.^{65–69} There are many differences between piRNAs and miRNAs. Firstly, the size of piRNAs is greater than the 21–23 nucleotide miRNAs, indicating a different biogenesis pathway. Indeed, production of piRNAs does not require Dicer activity.^{70,71} Secondly, piRNAs are most robustly expressed in the germline, whereas miRNAs are more broadly expressed. Thirdly, most piRNAs are generated from a small number of distinct loci termed piRNA clusters that are largely composed of transposon and repetitive sequences;⁷² in contrast, genes encoding miRNAs are found dispersed in animal genomes.

1.3.1 Mechanism for piRNA-mediated regulation of gene expression

Evidence supports a model in which piRNAs are processed from a single-stranded RNA precursor transcript. Because the transposon sequences in piRNA clusters are incomplete or damaged, they are not likely to be able to be expressed or mobilised. Like miRNAs, piRNAs interact and function with a protein in the Argonaute/Piwi (P-element-induced wimpy testis) family. Whereas Argonaute proteins in the miRISC are not catalytically active, all three Piwi proteins in flies (Piwi, Aubergine and Ago3) have Slicer activity and direct the cleavage of target RNAs between nucleotides 10 and 11.^{73,74} Ago3-bound piRNAs are derived from the sense strand of transposons, whereas Piwi and Aubergine-bound piRNAs are derived

from the antisense strand.^{72,73} After the processing of the primary transcript that is derived from a piRNA cluster, there is an amplification loop. Active transposon transcripts are recognised by antisense strand piRNA-containing Piwi/Aubergine complexes and cleaved, which after a second 3' cleavage event generates additional sense strand piRNAs that can then associate with Ago3. This self-reinforcing amplification loop has been termed the 'ping-pong model'.^{72,73}

Slicer activity by Piwi proteins generates the 5' end of piRNAs. Additional proteins are necessary for cleavage as well as for methylation at the 3' end of mature piRNAs. In flies, nucleases encoded by *zucchini* and *squash* genes appear to function in piRNA biogenesis as mutations in these genes result in reduced piRNA production and silencing of retrotransposons.⁷⁵ The RNA methyltransferase in flies, DmHen1/Pimet, functions to methylate the 3' end of piRNAs.^{76,77} However, unlike Piwi mutants, DmHen1/Pimet mutants are viable, indicating that this methylation event is not essential for piRNA activity.⁷⁷

The silencing of transposons is essential in the germline where the Piwi subfamily is primarily expressed in flies and mice,⁷⁸ although it is likely that additional functions exist for piRNAs in the soma.^{79–81} The Piwi protein was first described in 1997 in studies of germline development in flies, in which Piwi is necessary to maintain germline stem cells.⁸² Defects in the piRNA pathway results in overexpression of retrotransposons and DNA damage.⁷⁸ In mice, the three Piwi orthologues, MIWI, MILI and MIWI2, are all expressed in the testes and are required for spermatogenesis.⁷⁸ Mutations in any of the Piwi orthologues results in DNA damage and the induction of the apoptosis pathway in germline cells.^{83–85} DNA damage is likely due to a failure to silence transposons,⁸⁶ suggesting a conserved function of Piwi proteins and piRNAs in blocking DNA damage due to retrotransposon activity.

1.4 Summary and Perspectives

Even with the astonishing progress made in the last decade of research, the landscape of small RNA regulation of gene expression is still expanding. The biological activity of the majority of miRNAs and their critical target mRNAs are largely unknown. Chapters in this volume provide detailed

analysis of the current state of our knowledge of the functions of miRNAs and piRNAs during animal development and in the initiation or progression of human cancers.

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