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Review

Posttranscriptional Regulation of MicroRNA Biogenesis in Animals

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MicroRNAs (miRNAs) control gene expression in animals, plants, and unicellular eukaryotes by promoting degradation or repressing translation of target mRNAs. miRNA expression is often tissue specific and developmentally regulated, and regulation occurs both transcriptionally and posttranscriptionally. This regulation is crucial, as alteration of miRNA expression has been linked to human diseases, including several cancers. Here, we discuss recent studies that shed light on how multiple steps in the miRNA biogenesis pathway are regulated to modulate miRNA function in animals.

Introduction

The *lin-4* miRNA was identified in *C. elegans* in 1993 (Lee et al., 1993). At the time, *lin-4* was thought to be a worm-specific curiosity, but with the subsequent identification of the *let-7* miRNA, which is phylogenetically conserved (Pasquinelli et al., 2000; Reinhart et al., 2000), researchers took greater notice and began looking for other similar small RNAs. Since then, thousands of miRNAs have been found in animals, plants, and unicellular eukaryotes, and it is becoming clear that miRNAs have a big impact on shaping transcriptomes and proteomes of eukaryotic organisms (Baek et al., 2008; Lim et al., 2005; Selbach et al., 2008).

Conventional wisdom holds that miRNAs control the expression of specific genes, typically by base pairing to the 3′ untranslated regions (3′UTRs) of target messenger RNAs (mRNAs) to mediate repression of that target message, either by transcript destabilization, translational inhibition, or both (Filipowicz et al., 2008). However, recent studies have shown that translational inhibition alone is the exception rather than the rule, at least for mammalian miRNAs (Hausser et al., 2009; Hendrickson et al., 2009), and that miRNAs can also bind to other regions of a gene including the protein-coding exons (Rigoutsos, 2009). Computational and experimental approaches indicate that a single miRNA may target, on average, more than a hundred mRNAs. Furthermore, over 60% of human protein-coding genes are predicted to contain miRNA-binding sites within their 3′UTRs (Friedman et al., 2009). More than 700 miRNA genes have already been identified in the human genome alone, which approaches about ~3% of the number of all human protein-coding genes. Thus, miRNAs constitute one of the most abundant classes of gene-regulatory molecules in animals.

miRNAs are involved in a broad range of developmental and physiological processes (Bushati and Cohen 2007); thus, their deregulation is closely linked to human diseases, such as cancer (Chang and Mendell, 2007; Garzon et al., 2009). A large fraction of miRNAs exhibit strict developmental stage-specific and tissue-specific expression patterns, and the levels of many of these miRNAs are altered during disease. Although the final synthesis rate of a miRNA can, in principle, be controlled at any step of miRNA biogenesis, from transcription to mature miRNA turn over, recent findings have uncovered a significant role for posttranscriptional mechanisms in the regulation of miRNA biogenesis and activity (Carthew and Sontheimer, 2009; Davis and Hata, 2009). Here, we review recent progress in our understanding of the posttranscriptional mechanisms of miRNA biogenesis in animals.

General miRNA Biogenesis

Most mammalian miRNAs are transcribed by RNA rolymerase II (pol II), which generates a primary miRNA (pri-miRNA) transcript that consists of one or more hairpin structures, each composed of a stem and a terminal loop. Pri-miRNAs are 5′-capped, spliced, and polyadenylated, and they often produce more than one functional miRNA (Carthew and Sontheimer, 2009; Kim et al., 2009). Two subsequent, sequential processing reactions—one in the nucleus and one in the cytoplasm—trim the pri-miRNA transcript and generate the mature miRNA.

In the nucleus, the pri-miRNA is "cropped" into a ~70 nucleotide (nt) hairpin-structured precursor (pre-miRNA) by a multiprotein complex called the Microprocessor. The two core components of the Microprocessor are Drosha, an RNase III enzyme, and DGCR8/Pasha, a double-stranded RNA-binding domain (dsRBD) protein. The Microprocessor complex also contains a variety of cofactors including the DEAD box RNA helicases p68 (DDX5) and p72 (DDX17), as well as heterogeneous nuclear ribonucleoproteins (hnRNPs) (Gregory et al., 2004). These auxiliary factors may function to promote the fidelity, specificity, and/or activity of Drosha cleavage. DGCR8/Pasha functions at least in part by binding to the junction between single-stranded (ss) and double-stranded (ds) region of the pri-miRNA stem and directing Drosha to cleave ~11 bp away from the junction (Han et al., 2006). Drosha cleavage occurs cotranscriptionally, before splicing of the host RNA (Kim and Kim, 2007; Morlando et al., 2008), and generates a product with a 2 nt 3′ overhang, characteristic of RNase III-mediated cleavage. The overhang is recognized by Exportin-5, which transports the pre-miRNA into the cytoplasm via a Ran-GTP-dependent mechanism (Kim et al., 2009; Okada et al., 2009). In the cytoplasm, the pre-miRNA gets further "diced" into a ~22 nt long miRNA duplex by Dicer, another RNase III enzyme, in collaboration with the dsRBD

proteins TRBP/PACT (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006). The two miRNA strands are then separated and one of the strands associates with an Argonaute (AGO) protein within the RNA-induced silencing complex (RISC or miRISC) where it acts as a guide to repress target messages. The miRNA guides RISC to complementary sites within the target mRNAs to mediate repression of that target message.

While most miRNAs are generated by this pathway, some animal miRNAs are not. "Mirtrons," for example, are pre-miRNA-like hairpins that are made by splicing and debranching of short hairpin introns (Okamura et al., 2007; Ruby et al., 2007). Some small nucleolar RNAs (snoRNAs), transfer RNAs (tRNAs), and endogenous short hairpin RNAs (shRNAs) are also processed into miRNA-like molecules independently of the Microprocessor complex (Babiarz et al., 2008; Ender et al., 2008; Saraiya and Wang 2008; Cole et al., 2009). Deep sequencing efforts also reveal that endo-siRNA loci produce a distinct fraction of small RNAs that seem to load onto AGO1, and therefore might constitute functional miRNAs (Czech et al., 2009). Indeed, terminal hairpins of endogenous siRNA (endo-siRNA) long-stem-loop precursors are another source of miRNAs that bypass the Drosha processing step (Miyoshi et al., 2010).

The two strands of the miRNA duplex are not equal, and the inherent features of the duplex help to determine which strand is incorporated into RISC (Carthew and Sontheimer, 2009; Kim et al., 2009; Siomi and Siomi, 2009). One important parameter is the thermodynamic asymmetry of the miRNA duplex (Khvorova et al., 2003; Schwarz et al., 2003). Typically, the miRNA strand whose 5′ end is less stably base-paired will be more frequently chosen as the guide. In contrast, the miRNA strand whose 5′ end is more stably base-paired, also known as the passenger strand or the miRNA* strand, is excluded from the miRISC and generally thought to be degraded. In *Drosophila*, miRNAs and siRNAs are actively sorted into AGO1- and AGO2-RISC complexes, respectively, and AGO1 sorting involves Dicer-1/Loquacious (Loqs) whereas AGO2 sorting involves Dicer2/R2D2 (Siomi and Siomi, 2009). Central mismatches reduce binding of small RNA duplexes by the Dicer-2/R2D2 heterodimer, antagonizing AGO2 loading and promoting loading into AGO1 (Tomari et al., 2007). Recent deep-sequencing efforts, however, indicate that a large number of miRNA*s are not degraded, but rather associate with AGO1 or AGO2 (Czech et al., 2009; Ghildiyal et al., 2010; Okamura et al., 2008, 2009; Seitz et al., 2008). In such cases both miRNA and miRNA* strands are functional (Czech et al., 2009; Okamura et al., 2008, 2009). Differential sorting of these miRNA/miRNA* duplexes correlates with specific mismatches: Watson-Crick base-pairing at positions 9 and 10 promotes miRNA* sorting to AGO2 (Czech et al., 2009; Okamura et al., 2009). The identity of the 5′ nucleotide also affects strand selection, as observed in plants (Chen 2009; Czech et al., 2009; Okamura et al., 2009). Together, these findings suggest that miRNA precursors can be bifunctional, with individual strands adopting different fates within small RNA pathways.

In humans, all four AGO proteins show remarkably similar preferences for the structures of small RNA duplexes; central mismatches promote RISC loading, and thus their features are reminiscent of *Drosophila* AGO1 (Yoda et al., 2010). Although strand selection based on 5′ thermodynamic stability still contributes greatly to the sorting process, relative expression levels of the miRNA/miRNA* strands vary widely among tissues (Landgraf et al., 2007; Hu et al., 2009), indicating that strand selection factors other than sequence features might exist and that miRNA processing pathways are more complex than currently recognized.

Regulation of miRNA Maturation: Global and Individual

miRNA biogenesis is stringently controlled and is often subjected to feedback regulation. For example, some miRNAs regulate transcription factors that, in turn, regulate expression of the miRNA, forming a double-negative feedback loop (Li et al., 2009; Xu et al., 2009; for reviews, see Carthew and Sontheimer, 2009; Davis and Hata, 2009). This coordinated expression might serve to prevent misexpression of cell-type-specific miRNAs and to confer robustness to the underlying gene expression programs during development.

Interestingly, multiple steps of miRNA biogenesis are specifically regulated during processes like differentiation and tumor progression. For example, during early development, numerous pri-miRNAs are expressed but are not efficiently converted into mature miRNAs (Thomson et al., 2006). Similarly, reduced processing contributes to widespread downregulation of miRNAs in many human cancers (Garzon et al., 2009; Lu et al., 2005; Thomson et al., 2006). These findings have uncovered a significant role for posttranscriptional mechanisms of miRNA biogenesis.

Processing of individual miRNAs can also be independently regulated. For instance, many miRNAs are encoded in the genome as clusters and can be transcribed as long polycistronic primary transcripts; however, there is an example of one particular miRNA located in a cluster that is regulated independently from the other miRNAs in the cluster (Guil and Cáceres, 2007). This indicates that individual miRNAs can be posttranscriptionally regulated by factors that confer the specificity and function at various points during the biogenesis pathway. How is miRNA processing controlled at the posttranscriptional level? Proteins of known function or activity in posttranscriptional mechanisms of miRNA biogenesis are summarized in Table 1.

Regulation of miRNA Maturation at the Level of "Cropping"

Recent studies have shown that activity and specificity of Microprocessor-mediated pri-miRNA cleavage is subject to intense regulation. Adenosine-to-inosine (A-to-I) RNA editing of specific pri-miRNAs by ADAR (adenosine deaminase acting on RNA) enzymes prevents their effective processing by Drosha and, in some cases, A-to-I editing directs RNA molecules to Tudor-SN (Tudor staphylococcal nuclease homolog) for degradation (Yang et al., 2006). Editing in the miRNA "seed" region can also lead to the production of modified mature miRNAs that can potentially recognize a different set of targets compared with the unmodified miRNAs (Kawahara et al., 2007). Thus, RNA editing is an effective mechanism for modifying the biogenesis and activity of miRNAs.

Depletion of either of the RNA helicases p68 or p72, cofactors of the Microprocessor complex, results in a reduction in the

Table 1. Posttranscriptional Regulators of miRNA Biogenesis in Animals

Protein	Motifs	Known Activity	Mechanisms	Target miRNA	References
ADARs	dsRBD	A-I RNA editing	Inhibition of Drosha and Dicer processing	A subset	Yang et al. 2006; Kawahara et al. 2007
p68/p72	DEAD-box	Components of Microprocessor	Promoting Drosha cleavage	A subset	Fukuda et al. 2007
p53	DNA binding	Tumor suppressor	Binding to p68 and Promoting Drosha cleavage	miR-16-1, miR-143	Suzuki et al. 2009
SMADs	DNA binding	Signal transducers of TGFβ	Binding to p68 and Promoting Drosha cleavage	miR-21, miR-199a	Davis et al. 2008
ERα	DNA binding	Nuclear estrogen receptor	Binding to p68/p72 and inhibiting Drosha cleavage	A subset	Yamagata et al. 2009
hnRNP A1	RRM, M9	Pre-mRNA splicing	Chaperone for Drosha/DGCR8 binding	miR-18a	Guil and Cáceres, 2007
KSRP	KH	mRNA decay	Promoting Drosha and Dicer processing	A subset	Trabucchi et al. 2009
ARS2	Plant SERRATE homolog	Nuclear Cap-binding	Enhancing Drosha processing	Global	Gruber et al. 2009; Sabin et al. 2009
DGCR8	dsRBD	Binding to Drosha	Stabilizing Drosha	Global	Han et al, 2009
Exportin-5	RanBP	Binding to tRNAs and pre-miRNAs	Nuclear transport of pre-miRNA	Global	Grimm et al., 2006; Diederichs et al., 2008
LIN-28	CCHC-type zinc finger	Promoting pluripotency	Inhibition of Drosha and Dicer processing, and Recruiting TUT4	let-7	Heo et al. 2008; Newman et al 2008; Rybak et al. 2008; Viswanathan et al, 2008
TUT4	Poly(A) polymerase, CCHC-type zinc finger	Terminal uridylation	Binding to LIN-28 and inhibiting Dicer processing	let-7	Hagan et al. 2009; Heo et al. 2009
TRBP	dsRBD	Binding to Dicer, MKK phosphorylation site	Stabilizing Dicer	Global	Paroo et al., 2009
XRN-2	5′ to 3′ exoribonuclease	exoribonuclease	Degrading miRNA	Global	Chatterjee and Grosshans, 2009
GLD2	Poly(A) polymerase	Terminal adenylation	Stabilizing miRNA	miR-122	Katoh et al., 2009
mLin41	TRIM-NHL (RING finger)	Ubiquitinylation	Binding to Ago2 and targeting it for degradation	Let-7 and others in ES cells	Rybak et al., 2009
TRIM32	TRIM-NHL (RING finger)	ubiquitinylation	Binding to miRISC and enhancing miRNA activity	A subset	Hammell et al., 2009
NHL-2	TRIM-NHL (RING finger)	ubiquitinylation	Binding to miRISC and enhancing miRNA activity	A subset	Schwamborn et al., 2009
Mei-P26	TRIM-NHL (RING finger)	ubiquitinylation	Binding to miRISC and inhibiting miRNA activity	A subset	Neumüller et al., 2008
Argonautes	PAZ, PIWI	Components of RISC	Stabilizing associated miRNAs	Global	Diederichs et al., 2008

ADARs, adenosine deaminase acting on RNA enzymes; dsRBD, double-stranded RNA-binding domain; DGCR8, DiGeorge syndrome critical region gene 8; TGFβ, transforming growth factor β; ERα, estrogen receptor α; hnRNP A1, heterogeneous nuclear ribonucleoprotein A1; RRM, RNA recognition motifs; M9, nucleocytoplasmic shuttling signal sequence; KSRP, KH-type splicing regulatory protein; KH, hnRNP K homology domains; RanBP, ran-binding protein; ARS2, arsenic resistance protein 2; TUT4, terminal uridylyl transferase 4; TRIM-NHL, tripartite motif (consisting zinc fingers of both RING type and B Box type and a coiled-coil domain)-NHL repeats.

levels of a particular set of miRNAs (Fukuda et al., 2007), suggesting a role for p68 and p72 in promoting Drosha cleavage of a subset of miRNAs. p68 and p72 interact with a large number of proteins (Davis and Hata., 2009). The tumor suppressor protein p53, under conditions of DNA damage, is present in a complex with both Drosha and p68, and enhances Drosha processing of a subset of miRNAs, including miR-16-1 and miR-143 (Suzuki et al., 2009). Treatment of human vascular smooth muscle cells with either bone morphogenetic protein 4 (BMP4) or transforming growth factor beta (TGF-β) stimulates SMAD binding to p68 and increases Drosha processing of pri-miR-21 and pri-miR-199a (Davis et al., 2008). Estradiol (E2) treatment of ovariectomized female mice inhibits the production of a subset of miRNAs in the uterus through binding to its nuclear estrogen receptor α (ERα), which interacts with p68 and p72. ERα bound to E2 interacts with Drosha in a p68-/p72-dependent manner

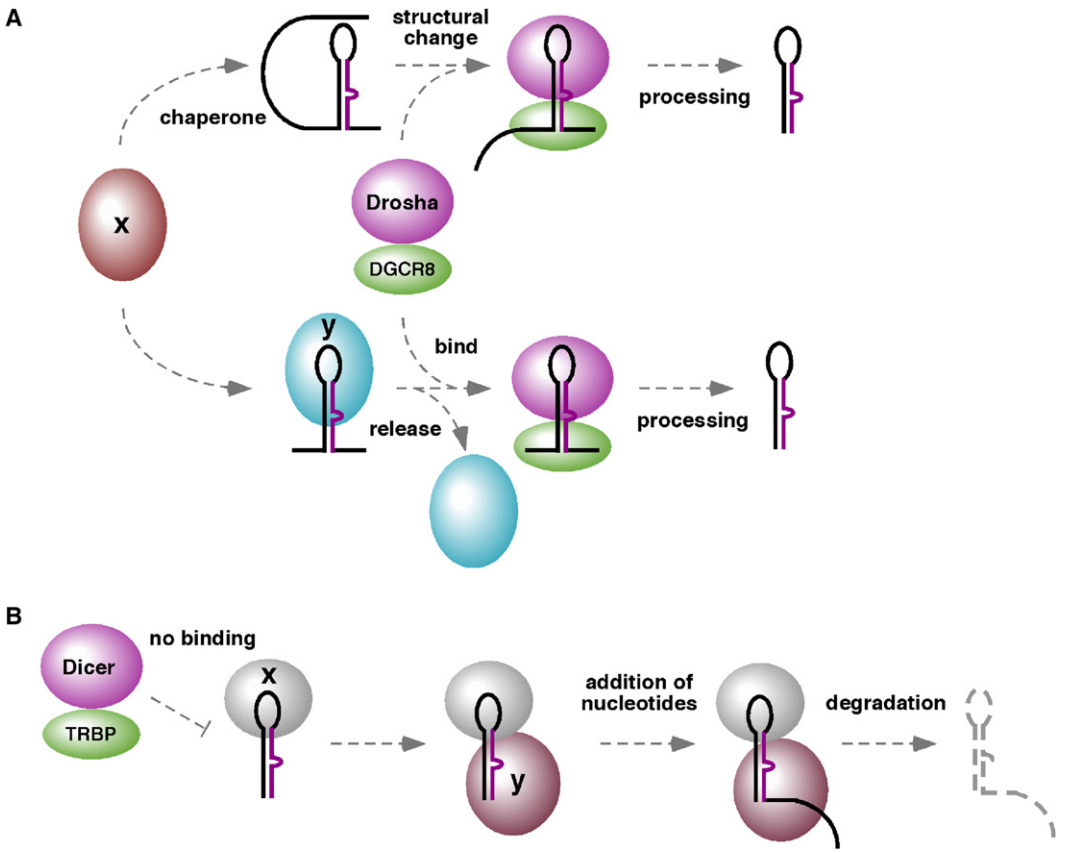

Figure 1. Posttranscriptional Controls of miRNA Biogenesis Mediated by RNA-Binding Proteins
The binding of RNA-binding proteins to miRNA precursors, particularly to the stem-loop structures within them, either blocks or enhances further processing by competing with or recruiting miRNA processing complexes. In (A), specific binding of RNA-binding proteins (X in the figure) to miRNA precursors can induce either a conformational change (chaperone activity; e.g., hnRNP A1) or a displacement of repressive RNA-binding proteins (Y in the figure).
(B) RNA-binding proteins (X in the figure) can also recruit auxiliary factors (Y in the figure) on specific miRNA precursors, thereby regulating processing, stability and subcellular location of miRNAs. For example, Lin28 competes with Dicer for pre-let-7. Upon binding by recognizing a specific sequence motif in the terminal loop, Lin28 recruits TUT4 to pre-*let-7*, leading to the 3′ terminal uridylation and the degradation of pre-let-7.

and appears to induce the dissociation of the Microprocessor complex from the pri-miRNAs or a conformational change of the Microprocessor with the consequence of reduced association prior to interaction with the pri-miRNAs, thereby blocking Drosha-mediated processing (Yamagata et al., 2009).

These observations indicate that signaling cascades regulate biogenesis of individual miRNAs and suggest that the interaction of p68 and/or p72 with accessory factors, such as p53 and SMAD proteins, can modulate specificity and activity of Drosha/DGCR8 processing, probably through a conformational change of the Microprocessor or pri-miRNAs or by a displacement of repressive RNA-binding proteins (Figure 1A). It will be important to identify factors that supply specificity to the Microprocessor complex for recognizing to a particular subset of pri-miRNAs and factors that supply specificity for selecting the Microprocessor complex on a particular subset of pri-miRNAs among all other Microprocessor complexes. However, an important recurring theme is that different signaling pathways can regulate the Microprocessor complex by modulating the activity of p68-/p72-interacting proteins.

Regulation at Cropping: The Importance of the miRNA Loop

miR-18a is processed from a polycistronic pri-miR-17-92 transcript, which harbors five additional miRNAs. hnRNP A1, an mRNA splicing regulator, exclusively stimulates maturation of miR18a from the miR-17-92 cluster. Pri-miR-18a, but not other members of the cluster, contains two regions of similarity to the consensus hnRNP A1-binding site, UAGGGA/U, within its terminal loop and stem (Guil and Cáceres, 2007; Michlewski et al., 2008). Direct and specific interaction of hnRNP A1 with the terminal loop and stem of the pri-miR-18a hairpin induces a structural rearrangement of the hairpin to generate a more favorable Drosha/DGCR8 binding and cleavage site (Figure 1A). Thus, hnRNP A1 acts as a chaperone for recognition and cropping of specific pri-miRNAs by Drosha/DGCR8. Interestingly, approximately 14% of human miRNAs contain highly conserved loop sequences, suggesting that processing regulation by hnRNPs and other nuclear RNA-binding proteins might extend well beyond miR-18a (Michlewski et al., 2008). Indeed, the KH-type splicing regulator protein (KSRP, also known as KHSRP)

recognizes G-rich regions, including a GGG triplet, present within the terminal loops of a subset of pri-miRNAs, to promote Drosha/DGCR8 mediated processing (Trabucchi et al., 2009). Upon binding, KSRP could optimize the positioning and/or recruitment of the miRNA precursor processing complexes through protein-protein interactions. KSRP is associated with the terminal loop of the target miRNA precursors during nucleocytoplasmic transit and also promotes the processing of the target pre-miRNAs by Dicer in the cytoplasm. This further suggests that specific recognition of the terminal loop by RNA-binding proteins is an important means to extend regulation of miRNA processing down to the level of individual miRNAs.

Binding of RNA-binding proteins to specific sequences present in the terminal loop regions of miRNA precursors potentially either competes for binding or promotes recruitment of processing factors. Many signaling pathways operate by modifying the activity of specific RNA-binding proteins, in which RNA-binding motifs are combined with other conserved domains, such as protein-protein interaction domains and consensus phosphorylation motifs (Davis and Hata, 2009). Changes of expression, RNA-binding activity, interacting protein partners, and subcellular localization of RNA-binding proteins in response to extracellular signals might serve to regulate biogenesis of individual miRNAs.

Global Regulation: Possible Links to Other Nuclear RNA Processing Events

Accumulation of global miRNA with increasing cell density has been observed and is associated with enhanced Drosha processing activity without changing protein levels of Drosha or DGCR8 (Hwang et al., 2009). This suggests that miRNA biogenesis is linked to the proliferative state of the cell and that processing activity can be regulated for the same stoichiometoric amount of the complex by altering turnover rate of the single Microprocessor complexes. Arsenate resistance protein 2 (ARS2), whose expression is low during cell quiescence and high during proliferation, is a component of the nuclear RNA cap-binding complex (CBC) that binds and stimulates the activity and fidelity of the Microprocessor complex (Gruber et al., 2009; Sabin et al., 2009). Interestingly, the plant homolog of ARS2, SERRATE (SE), is an essential factor in miRNA biogenesis that interacts with the nuclear CBC and RNase III enzyme Dicer-like 1 (DCL1), the main miRNA-processing enzyme in plants (Chen, 2009). The nuclear CBC containing ARS2 may function to link pri-miRNA processing to other RNA processing events. For example, CBC/ARS2 may recruit the Microprocessor to 5′-capped nascent pri-miRNA transcripts, as seen for SE in plants, and might also either stabilize pri-miRNAs or enhance the cleavage activity and specificity of Drosha/DGCR8. Rapid cytoplasmic transport of pri-miRNA transcripts may compete with Drosha/DGCR8 recognition and cleavage in the nucleus. Together, CBC and ARS2 may counteract this transport by repressin nuclear export of pri-miRNA transcripts, thereby increasing the opportunity for Microprocessor recognition and processing of target hairpins. Furthermore, approximately 40% of miRNAs are encoded in the introns of genes (Kim et al., 2009) and therefore some Pol II transcripts are simultaneously pre-mRNAs and pri-miRNAs. This suggests that there may be physical connections between the Microprocessor and the pre-mRNA splicing machinery as SE, like hnRNP A1 and KSRP, also acts in pre-mRNA splicing. The CBC not only recruits export factors to nascent pre-mRNAs, but also interacts directly with splicing factors (Pawlicki and Steitz, 2010) and may, therefore, play an integral role in the coordination of pre-mRNA and pri-miRNA processing. Once processed, pre-miRNAs are transported to the cytoplasm by Exportin-5, which appears to be limiting (Grimm et al., 2006; Diederichs et al., 2008). Thus pre-miRNA transport can also be regulated.

Autoregulation of the miRNA Cropping Machinery

Drosha and DGCR8 are also tightly regulated in an intricate feedback circuit, which might contribute to the homeostatic control of miRNA biogenesis (Han et al., 2009). DGCR8 stabilizes Drosha through a direct interaction. In turn, Drosha cleaves two hairpin structures in the 5′UTR and the coding sequences of the DGCR8 mRNA, whose folds are similar to pri-miRNA structure, thus leading to degradation of the mRNA. This results in a double-negative feedback loop, ensuring tight coupling of the core Microprocessor components. A similar feedback mechanism regulates pri-miRNA processing in plants (Rajagopalan et al., 2006). DICER-LIKE1 (*DCL1)* pre-mRNA harbors the pre-miR838 hairpin. DCL-mediated processing of *DCL1* pre-mRNA releases pre-miR838, generating nonproductive fragments of *DCL1* pre-mRNA. It is likely that animal cells may (de)stabilize Drosha by modifying its interacting partner DGCR8 or by masking pri-miRNA-like hairpins in the DGCR8 mRNA by recruiting an RNA-binding protein. This would alter global miRNA biogenesis in response to the proliferative state of the cell or to extracellular stimuli. Although analysis of deep-sequencing data of small RNAs in mouse cells suggests that the Microprocessor's role in directly destabilizing coding mRNAs may be only targeted to DGCR8 mRNA itself (Shenoy and Blelloch, 2009), it is still tempting to speculate that some mRNAs with pri-miRNA-like hairpins could be involved in a network that balances global miRNA biogenesis by acting as attenuators that compete with authentic pri-miRNAs for Microprocessor binding.

Regulation of Dicer Activity

Pre-miRNA processing by Dicer appears to function quite efficiently since pre-miRNA levels are low relative to the levels of pri- and mature miRNA, suggesting that dicing is a mechanism with rapid substrate turnover and very little regulation. However, recent studies have shown that the expression and activity of Dicer might serve as an important regulatory point in global and individual miRNA biogenesis. Dicer activity is repressed by the nuclear retention of pre-miRNAs that prevents access by cytoplasmic Dicer (Lee et al., 2008). Dicer activity might also be repressed by the presence of a specific inhibitor that can compete with Dicer for pre-miRNA binding, though such an inhibitor has not yet been identified (e.g., pre-miR-138 in mouse brain) (Obernosterer et al., 2006). The levels of Dicer can be regulated by its products; the identification of let-7 targeting sites within Dicer coding sequence suggests that let-7 miRNA directly targets Dicer, thus establishing a miRNA/Dicer autoregulatory negative feedback loop that might regulate the total rate of miRNA biogenesis (Forman et al., 2008). In *C. elegans*, Dicer

activity is inhibited by *rncs-1*, a highly base-paired 800 nt noncoding RNA, presumably by binding to Dicer or accessory dsRBD proteins to compete with endogenous dsRNAs involved in silencing (Hellwig and Bass, 2008).

Regulation at Dicing: The Importance of the miRNA Loop

The human *let-7* miRNA family is derived from 12 precursor transcripts that contain nine mature miRNA sequences. While most *let-7* pri-miRNA transcripts are highly expressed throughout development, the mature *let-7* is detectable only in highly differentiated cells, suggesting a posttranscriptional regulation of biogenesis (Roush and Slack, 2008). Levels of LIN-28, an RNA-binding protein that promotes pluripotency, inversely correlates with mature *let-7* expression during development (Wu and Belasco, 2005). Although LIN-28 was then found to block accumulation of mature *let-7* by repression of both Drosha and Dicer activities (Heo et al., 2008; Newman et al., 2008; Rybak et al., 2008; Viswanathan et al., 2008), recent studies demonstrate that LIN-28 can induce 3′ terminal uridylation of pre-*let-7* miRNA in mouse embryonic stem (ES) cells to block Dicer cleavage and *let-7* maturation (Heo et al., 2008). LIN-28 recruits a noncanonical poly (A) polymerase (PAP), terminal uridylyl transferase 4 (TUT4, also known as ZCCHC11 in mammals and PUP-2 in *C. elegans*) to pre-*let-7* by recognizing a tetra-nucleotide sequence motif (GGAG) in the terminal loop (Hagan et al., 2009; Heo et al., 2009) (Figure 1B). TUT4 then adds a uridine (U) tail to the 3′ end of pre-miRNA in a template-independent manner. Dicer is unable to cleave hairpin RNAs with such long 3′ extensions. Uridylyl groups are known to recruit 3′-to-5′ exonucleases, suggesting that 3′ terminal uridylation may also facilitate the degradation of pre-let-7 with a U tail. Several additional miRNAs that contain the GGAG motif in the terminal loop also undergo uridylation in a LIN-28-dependent manner (Heo et al., 2009). This suggests that the ability of LIN-28 and TUT4 to repress pre-miRNA processing by inducing uridylation might not be specific to let-7. Besides LIN28, other specificity factors may also assist TUT4 (and other TUTases) to recognize distinct pre-miRNAs. These findings suggest that the terminal loop is a pivotal structure or landing pad where miRNA processing ''activators'' (for example, hnRNP A1 and KSRP) as well as ''repressors'''(for example, LIN-28) function in a coordinated way to transform proliferating, apoptotic, or differentiating cues into changes of miRNA expression (Figure 1B). In *C. elegans,* LIN-28 stimulates uridylation of let-7 pre-miRNA by PUP-2, indicating that LIN-28 and let-7 form an ancient regulatory switch, conserved from nematodes to humans (Lehrbach et al., 2009). However, the terminal loop of pre-let-7 is not conserved in *C. elegans*. Thus, the exact mechanistic details might differ between organisms.

Signal-Mediated Regulation of Dicer Activity

Dicer protein levels can be regulated by TRBP through a direct interaction. Truncation mutations of TRBP that cause diminished TRBP protein expression are found in sporadic and hereditary carcinomas, and are associated with both a defect in the processing of miRNAs and in destabilization of the Dicer protein (Melo et al., 2009). The mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) pathway, which promotes cell growth, phosphorylates TRBP and enhances miRNA production by stabilizing the Dicer-TRBP complex (Paroo et al., 2009). The ERK signaling pathway involves a cascade of phosphorylation events from a MAPK kinase kinase, to a MAPK kinase (MKK), to MAPK ERK. TRBP interacts with phosphorylated ERK1/2 and its phosphorylation requires MKK1 and ERK2. Interestingly, phosphorylation of TRBP is blocked by the MKK inhibitor U0126, and concomitantly, the level of growth-promoting miRNA decreases and that of growth-suppressing miRNAs increases in cancer cell lines treated with U0126. These findings indicate a concerted miRNA regulatory program capable of responding positively to mitogenic signals and negatively following inhibition of these signals. This also implies that the level of the Dicer-TRBP complex determines preferential substrates for pre-miRNA processing, which might account for the observed decrease of growth-suppressing miRNAs in some cancer cells. How the level of the Dicer-TRBP complex determines processing of preferential target pre-miRNAs remains to be elucidated; however, these findings also imply therapeutic significance.

Regulation of miRNA Accumulation at the Level of miRNA Turnover

Maintaining proper steady-state levels of miRNAs is crucial for normal development and overexpression or underexpression of miRNAs are often linked to various human diseases. The steady-state levels of miRNAs are presumably determined by the opposing activities of miRNA biogenesis (transcription and processing) and degradation. In plants, a family of 3′ to 5′ exoribonucleases encoded by the *SMALL RNA DEGRADING NUCLEASE (SDN)* genes degrades mature miRNAs to limit their accumulation (Ramachandran and Chen, 2008). In *C. elegans*, degradation of mature miRNAs is mediated by the 5′ to 3′ exoribonuclease XRN-2, a tRNA quality control factor that selectively removes incompletely modified tRNAs in yeast (Chatterjee and Grosshans., 2009). Although miRNAs in the cell are present as miRISC complexes, in which both the 5′ and 3′ ends are thought to be bound directly by Argonaute (Wang et al., 2008) and therefore resistant to exonucleases, cells apparently have some way of dislodging miRNAs from Argonaute to expose the miRNAs to XRN-2 for degradation. miRNA degradation by XRN-2 can be blocked by the addition of miRNA target RNA, suggesting that targets can modulate the stability of individual mature miRNAs. These findings demonstrate that miRNA degradation affects functional miRNA homeostasis, helping to prevent detrimental overexpression of miRNAs. A coordination of miRNA and target levels could permit miRISC reprogramming or turnover when target abundance is low.

The stability of individual mature miRNAs can also be regulated. For example, the cytoplasmic poly(A) polymerase GLD2 (also known as TUTase2) specifically monoadenylates and stabilizes miR-122 in mammalian liver cells (Katoh et al., 2009). On the other hand, the addition of nontemplated adenines has been detected on many different animal miRNAs (Landgraf et al., 2007; Azuma-Mukai et al., 2008). This suggests that despite their small size, specific miRNAs contain additional sequence elements that control their behavior, probably through specific recognition by RNA-binding protein cofactors, thereby affecting miRNA turnover (Ambros, 2008; Hwang et al., 2007).

The protein factors governing the abundance of mature miRNAs also include Argonaute proteins. Increased levels of Argonaute proteins in mammalian cells correlate with increased levels of mature miRNAs (Diederichs et al., 2008). This effect depends on direct binding of the Argonaute proteins to the miRNA, suggesting that Argonaute proteins are limiting and serve to stabilize miRNAs. This coordinated regulation could provide a feedback mechanism to titrate the expression of mature miRNAs with the availability of Argonaute proteins to form functional, stoichiometric miRISCs. Therefore, regulation of the total levels of the Argonaute proteins within the cell contributes to global miRNA turnover. One such regulator is the type I collagen prolyl-4-hydroxylase (C-P4H[I]). Human AGO2 interacts with and is hydroxylated by the C-P4H(I), which enhances the stability of AGO2 (Qi et al., 2008). C-P4H(I) is upregulated by a variety of factors, including hypoxia and TGF-β, factors which also induce many miRNAs. Another example is the let-7 target gene mouse *Lin41* which encodes a member of the TRIM protein family that contains a RING finger domain with E3 ubiquitin ligase activity. mLin41 facilitates polyubiquitination of Ago2 in ES cells and targets it for degradation, suggesting that mLin41 is a negative regulator of cellular Ago2 levels. This provides evidence of an additional negative feedback loop that acts in addition to LIN-28 to regulate let-7 activity in mouse ES cells (Rybak et al., 2009). Similarly, other TRIM family proteins such as fly Mei-P26, mouse TRIM32, and worm NHL-2 were recently found to enhance or suppress RISC loading and RISC activity by binding to core miRNP components, including Ago proteins (Neumüller et al., 2008; Hammell et al., 2009; Schwamborn et al., 2009). Finally, formation and turnover of miRISC appears to depend on the endosomal pathway, where factors required for endosome formation also regulate miRNA loading into RISC (Gibbings et al., 2009; Lee et al., 2009). Therefore, turnover of the endosome might also affect the net accumulation of mature miRNAs.

Perspectives

As described above, final mature miRNA expression will depend on the cooperation of multiple mechanisms and their net effect. It is also becoming apparent that miRNA strand selection can be seen as a form of posttranscriptional regulation. The identification of factors that regulate both global and individual miRNA biogenesis and turnover represents an important challenge for future studies. It is important to bear in mind that miRNAs are part of a large multimolecular complex, miRISC, and their precursors associate with multiple RNA-binding proteins. Thus, one major future challenge will be to understand in more detail how RNA-binding proteins influence the final outcome of miRNA biogenesis and how they impose specificity on the expression and function of individual miRNAs. Thus, it will be critical to develop novel biochemical approaches to identify complexes containing miRNA, their precursors, and associated proteins in vivo. The HITS-CLIP (high-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation) method recently developed to decode miRNA-mRNA interactions (Chi et al., 2009) can be applied to the identification of factors that regulate miRNA biogenesis. The budding yeast model for RNAi (Drinnenberg et al., 2009) could also be used as a tool to study and genetically screen the pathways that regulate miRNAs. For this, it is probably worth determining if miRNA biogenesis can be reconstituted in *S. cerevisiae* by expressing human Dicer, TRBP, and AGO2 or their homologs of the unicellular green alga *Chlamydomonas reinhardtii,* which encodes many miRNAs (Molnár et al., 2007; Zhao et al., 2007). The study of posttranslational modification of both miRNA processing factors and their accessory factors might allow the identification of upstream signaling pathways that regulate and fine-tune the miRNA machinery. Further investigation should unravel multiple mechanisms and the interactions occurring among them that regulate and coordinate the precise expression of many miRNAs at posttranscriptional levels. Such knowledge will not only expand our understanding of how they are altered in pathological conditions but will present exciting opportunities for screening of compounds to manipulate aspects of the miRNA machinery for therapeutic application.

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