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A developmental view of microRNA function

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MicroRNAs (miRNAs) are genomically encoded small non-coding RNAs that regulate flow of genetic information by controlling translation or stability of mRNAs. Recent recognition that many miRNAs are expressed in a tissue-specific manner during development of organisms, from worms to humans, has revealed a novel mechanism by which the proteome is regulated during the dynamic events of cell-lineage decisions and morphogenesis. Advances in the understanding of miRNA biogenesis, target recognition and participation in regulatory networks demonstrate a role for miRNAs in lineage decisions of progenitor cells and organogenesis. Future discoveries in this area are likely to reveal developmental-regulation and disease mechanisms related to miRNAs.

Introduction

In metazoans, early embryonic patterning and organ morphogenesis involve coordinated differentiation, migration, proliferation and programmed cell death. These complex cellular and developmental processes depend on precise spatiotemporal regulation of mRNA and protein levels of key regulatory factors. The dose-sensitivity of proteins involved in morphogenesis is highlighted by the numerous human diseases caused by heterozygous mutations that result in haploinsufficiency. Protein levels can be controlled at multiple stages, including mRNA transcription and translation, and protein stability and degradation.

Animal species use evolutionarily conserved mechanisms, ranging from signaling events to chromatin remodeling and transcriptional regulation, to execute developmental programs. In recent years, evidence has accumulated that small non-coding RNAs are also used in a conserved manner to regulate key developmental events. At least four classes of regulatory small non-coding RNAs have been described, including microRNAs (miRNAs), short interfering RNAs (siRNA), repeat-associated small interfering RNAs (rasiRNAs) and piwi-interacting RNAs (piRNAs) [1,2]. Among these small RNAs, miRNAs are the most phylogenetically conserved and function post-transcriptionally to regulate many physiologic processes, including embryonic development [3–7].

The first known animal miRNA, lin-4, was discovered in a screen of *Caenorhabditis elegans* heterochronic genes, which distinguish one larval development stage from another. Analysis of the heterochronic pathway then led to the identification of the first miRNA target, lin-14 [3,4]. The discovery that another miRNA, let-7, is conserved from worms to mammals [5,8] resulted in the realization that miRNAs represent a widely used mechanism to regulate transfer of genetic information in almost all species. Over the past few years, >400 miRNAs have been identified, and there are probably many more still undiscovered [9–14]. Systematic analysis of the spatial expression of miRNAs has shown that many miRNAs are expressed in a tissue-specific manner [15,16]. Because each miRNA targets a large number of mRNAs for translational inhibition or degradation, it is likely that much of the transcriptome is regulated by miRNAs. Given the likely role of miRNAs in 'fine-tuning' protein dosage, they might represent an efficient system by which a cell can rapidly control threshold-dependent cellular events.

Adoption of cell lineages during embryonic development and subsequent morphogenetic events are mainly achieved by rapid and regional regulation of morphogen gradients that subsequently titrate transcriptional events during discrete windows of time. As a result, miRNAs might have a fundamental role in many, if not most, crucial embryonic decisions. During the past few years, several examples of miRNA regulation of developmental events have emerged that support such a notion. Progress in understanding the transcriptional regulation of miRNAs and identification of their targets provides an opportunity to dissect the complex networks involved in regulating protein dosage during cell-fate decisions and further embryogenesis. Here, we review recent conceptual advances in the transcriptional- and post-transcriptional regulation of miRNAs, their target recognition and the mechanisms by which miRNAs might regulate developmental events. Although the paradigms described here are relevant to miRNA biogenesis and function in other settings, owing to space constraints, we cannot review all aspects of miRNA biology but refer readers to several outstanding recent reviews [17–23].

Overview of the generation and function of miRNAs

A brief review regarding general features of miRNA biogenesis and function is provided here, which will be relevant to later discussions of developmental-specific miRNA biology. Numerous reviews provide more details regarding miRNA biogenesis (see, for example, Refs [18,23–26]).

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miRNA biogenesis and the RNA-induced silencing complex

miRNAs comprise 1–3% of the genome and are ~21 nucleotides (nt) in length in their mature form. They are generated via a multi-stage process involving both the nuclear and cytoplasmic compartments (Figure 1). The process culminates in their inclusion in a protein complex known as the RNA-induced silencing complex (RISC). The RISC can then repress mRNA translation or destabilize mRNA transcripts (Figure 1), thus leading to rapid silencing of the gene product. In human cells, mRNAs that are targeted for silencing by miRNAs – together with Argonaute proteins, the core component of RISCs – are concentrated in cytoplasmic foci called processing bodies (P-bodies) [23–26]. Typically, the complementarity between animal miRNAs and their targets is highest in the 5′ region of the miRNA, and, through mechanisms still poorly understood [20, 27–30], this interaction results in disruption of translation. Recent results indicate that miRNAs can also direct target mRNA degradation via a mechanism distinct from siRNA-directed endonucleolytic cleavage, but the details of this process are unknown [23–26,31] (Figure 1).

Genomic organization of miRNAs

The genomic organization of miRNAs is diverse. In some cases, miRNA genes contain their own independent promoters and enhancers (Figure 2). In humans and mice, ~40% of miRNAs are located within the introns of either non-protein-coding or protein-coding transcription units, and 10% are within exons. When orientated in the same direction as the surrounding gene, the miRNA is typically derived from the larger transcript in which it is embedded. A further ~30% have uncertain transcriptional origins, and the rest are derived from genomic repeats [32].

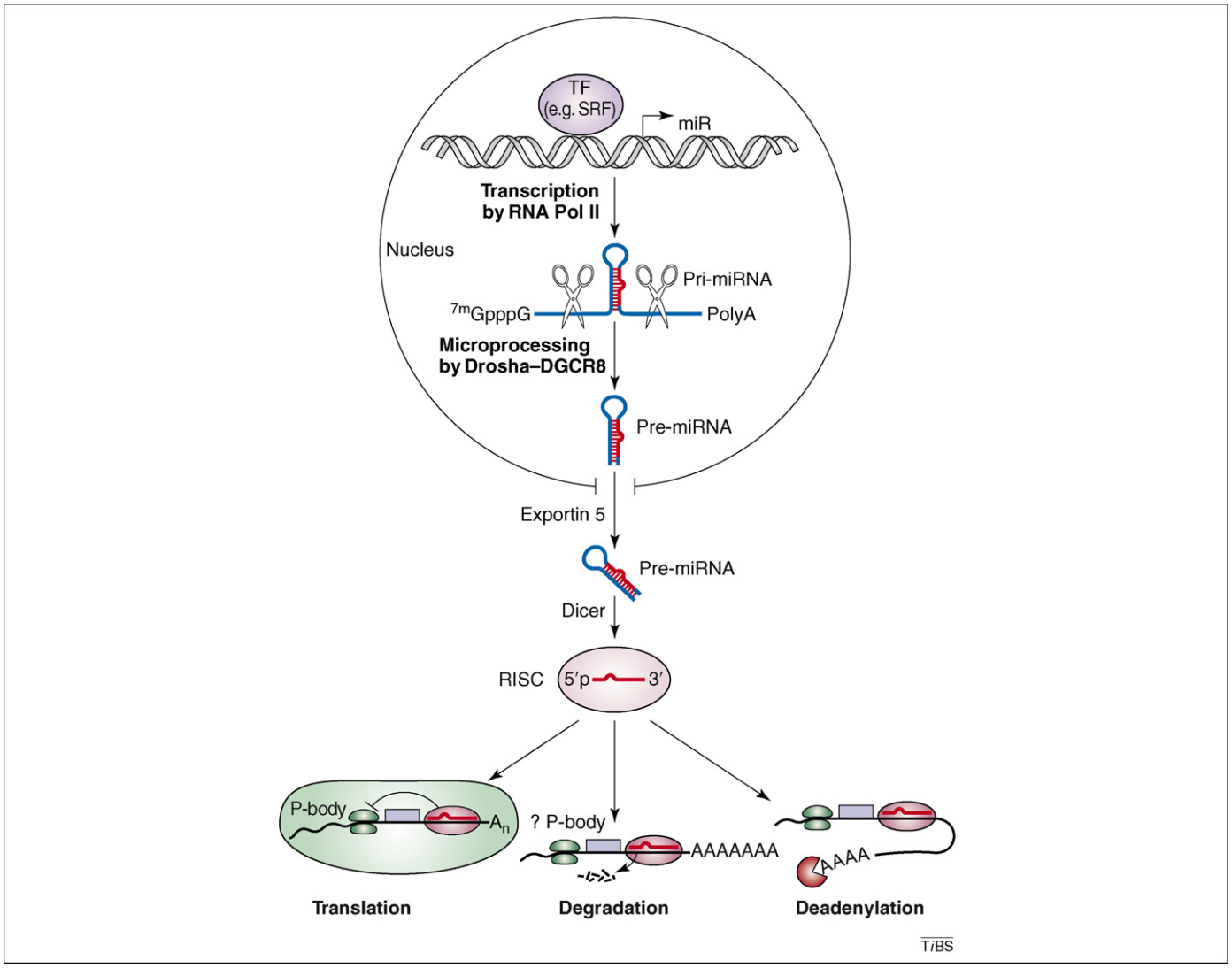

Figure 1. Current model of miRNA biogenesis and function. The initial RNA is typically transcribed by RNA polymerase II (pol II) as primary miRNAs (pri-miRNAs), which range from a few hundred to thousands of nucleotides (nt) in length. The pri-miRNA of each miRNA has a characteristic stem–loop structure that can be recognized and cleaved by the ribonuclease III (RNase III) endonuclease Drosha within the nucleus. Efficient pri-miRNA cleavage by Drosha requires a protein partner, DGCR8 (DiGeorge syndrome critical region gene; also known as Pasha), which has a double-stranded RNA-binding domain (dsRBD). The cleavage product, a ~70-nt stem–loop pre-miRNA, is exported from the nucleus to the cytoplasm by Exportin 5. In the cytoplasm, another evolutionarily conserved RNase III enzyme, Dicer, together with its dsRBD protein partner, *trans*-activation response (TAR) RNA-binding protein (TRBP) and PKR (RNA-dependent protein kinase)-activating protein (PACT), further process pre-miRNA into mature miRNA (~21 nt). The mature miRNA is then unwound and a single strand is incorporated into the RISC, which represses mRNA translation or destabilizes mRNA transcripts through cleavage or deadenylation [18,23–26]. Abbreviations: SRF, serum response factor; TF, transcription factor.

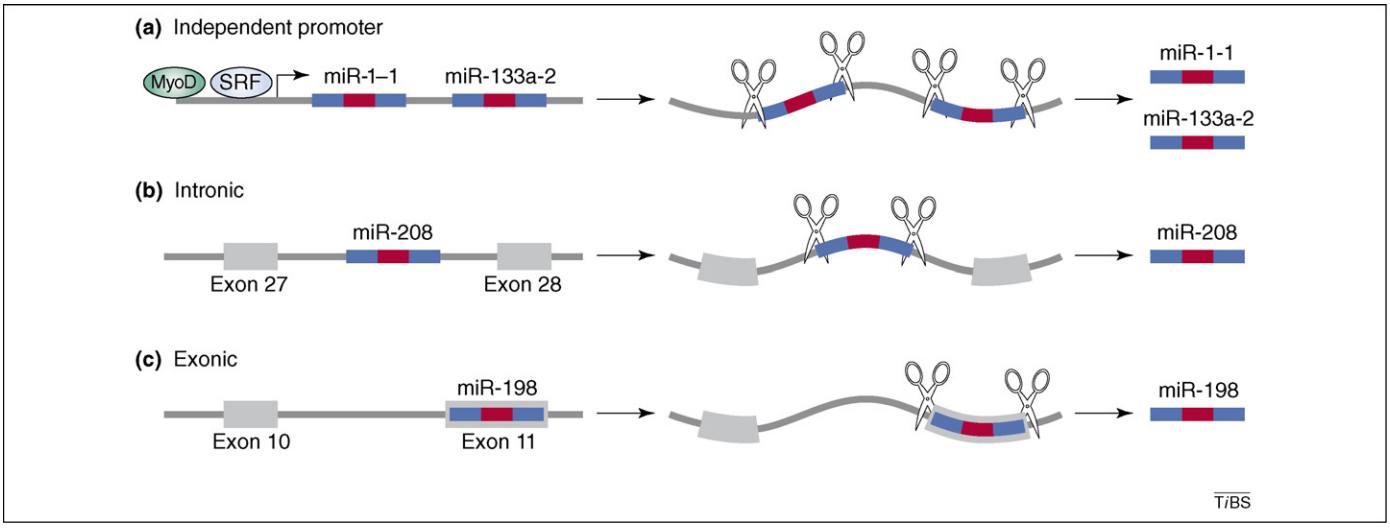

Figure 2. Genomic organization of miRNAs. miRNAs can exist in many different types of genomic loci. **(a)** Transcription of miRNAs can be independently regulated by separable promoters, as is seen for miR-1–1 and miR-133a-2, which are regulated by the transcription factors SRF and MyoD. Such miRNAs might still be located in introns but often have an antisense orientation. **(b)** Most intronic miRNA precursors have the same orientation as their host gene and are initially transcribed as part of its precursor RNA. For example, miR-208, which is expressed specifically in the mouse and human heart, resides in intron 28 of human cardiac α-myosin heavy chain. Intronic pri-miRNAs might be produced upon cleavage of the spliced intron by the Drosha endonuclease. **(c)** With few exceptions, miRNAs that are embedded in, or overlap with, exons of known transcripts are always in the same orientation, and most of these known transcripts are in the non-coding 5′ or 3′ UTRs (e.g. miR-198 in follistatin-like 1) [86]. Approximately 50% of miRNAs are in miRNA clusters that are initially encoded as a polycistronic transcript that is subsequently cleaved into multiple miRNAs. In most cases, polycistronic miRNAs share the same expression pattern. However, relative levels of miRNAs within the cluster seem to be regulated in a developmental and homeostatic manner, suggesting yet undescribed complexity of expression regulation. The genomic location of the miRNA sequence is indicated: blue, pre-miRNA sequence; red, mature sequence.

Transcriptional regulation of miRNAs

Although miRNAs can have spatiotemporally specific expression patterns, insight into their *in vivo* transcriptional regulation came from a recent analysis of the miR-1 family [7]. miR-1 is one of the most evolutionarily conserved miRNAs, with the mature form being identical in worms and humans [7,9,11]. The worm and fly genomes contain a single miR-1; fish, chick, mouse and humans each have two miR-1 genes, miR-1–1 and miR-1–2, which encode identical mature miRNAs. miR-1s are uniquely expressed in the developing heart and skeletal muscle progenitors and persist in the adult muscle.

Serum response factor (SRF), myocyte enhancer factor 2 (Mef2) and MyoD, which are all master regulators of myogenic differentiation, are required for regulation of miR-1–1 and miR-1–2 promoter regions *in vivo* and *in vitro*. SRF binds to and activates the promoter regions, and mice with targeted SRF deletion in the heart lack endogenous miR-1–1 and miR-1–2 expression. Similar findings support the idea that miR-1s are direct *in vivo* targets of MyoD and Mef2 in skeletal muscle [7]. The transcription factor binding sites seem to be ancient because they are conserved even in fruit flies, where an SRF-like site is essential for cardiac expression, and the basic helix–loop–helix (bHLH) transcription factor twist and mef2 regulate somatic muscle expression of miR-1 [7,33–35] (Figure 3).

Post-transcriptional regulation of miRNAs

The biogenesis of miRNAs, which involves initial transcription, processing and export from the nucleus, and further processing into the mature form of miRNA, seems to be regulated at multiple steps [36,37]. For example, miR-1 and miR-133 share common *cis*- and *trans*-regulation mechanisms, but the relative abundance of miR-1 or miR-133 differs dynamically in the heart and skeletal muscle at distinct stages of development, which reflects a higher order of processing regulation [7,38]. Likewise, the mature form of miR-38 in worms can only be detected in the embryo, although the pre-miR-38 transcript is uniformly expressed in embryonic and adult stages [39]. More strikingly, the mature forms of miRNAs derived from polycistronic clusters sometimes have unrelated expression patterns, as observed with the miR-23b–miR-27b–miR-24/miR-189 cluster and the miR-132–miR-212 cluster [40]. The recent discovery that RNA-editing enzymes can alter the cleavage site in pri-miR-142 within hematopoietic cells and thereby regulate the processing of miR-142 is exciting, and might represent a mechanism that is more widely used to control miRNA activity [41].

Target recognition: miRNA-seed pairing and RNA accessibility

The biology of miRNA function will be dictated by the mRNA transcripts targeted by specific miRNAs. However, it has been difficult to predict miRNA targets by nucleotide-sequence matching because of limited sequence complementarity even in the setting of efficient translational inhibition. There is good evidence that a high degree of complementarity with the 5′ end of the miRNA, particularly nucleotides 2–7, is important in target-mRNA recognition [27–29]. However, even among these six nucleotides, non-matching still occurs *in vivo*, which makes it difficult to use sequence matching as a predominant criterion for target prediction. Several computational programs take a bioinformatics approach to identify miRNA targets, but the number of targets predicted for a given miRNA ranges from 100 to 500, making validation studies difficult. Listed

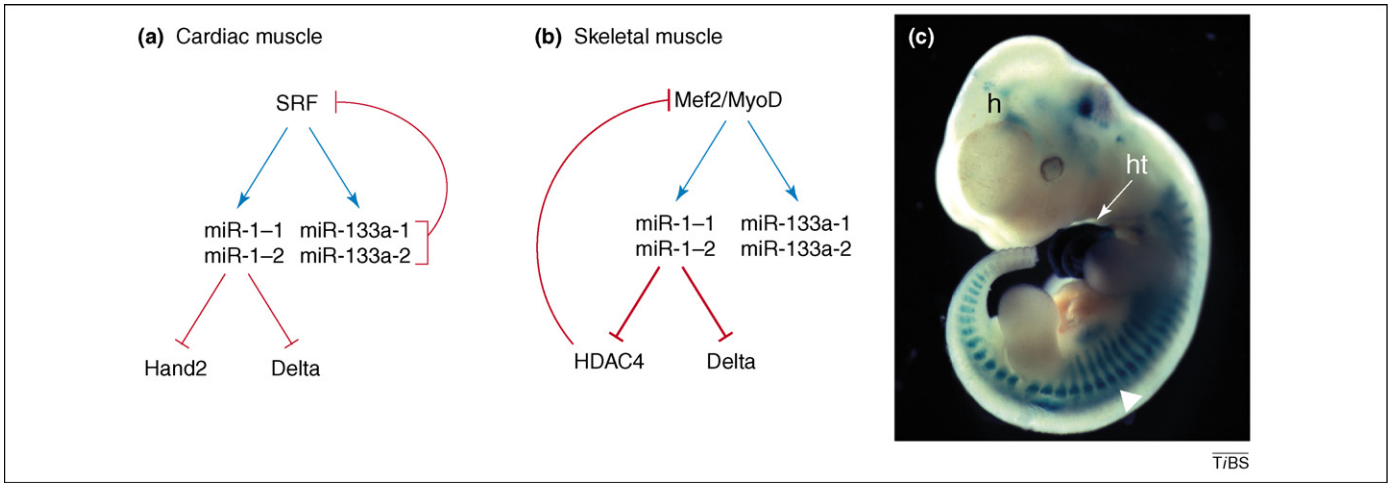

Figure 3. Role and regulation of muscle miRNAs. Simplified schematics of the current understanding of miR-1 and miR-133a pathways in **(a)** cardiac and **(b)** skeletal muscle are shown. (a) In cardiac myocytes, the activation of SRF induces the expression of miR-1–1 and miR-1–2, which then represses expression of the transcription factor Hand2 and the Notch ligand, Delta, thereby affecting progenitor expansion or differentiation. SRF also induces miR-133a-1 and miR-133a-2 expression, which inhibits SRF in a feedback loop. (b) A similar pathway operates in skeletal muscle, except that feedback inhibition of Mef2 and MyoD occurs when HDAC4 expression is reduced due to silencing by miR-1–1 and miR-1–2. **(C)** lacZ expression in the heart (ht) and somites (white arrowhead) is directed by the upstream enhancer of the miR-1–1 or miR-133a-2 cluster, indicating a role in heart and skeletal muscle development. Abbreviation: h, head. Part (c) adapted, with permission, from Ref. [46].

in Table 1 are examples of functionally characterized miRNA targets, which remain limited in number [42].

Not all mRNA targets with sequences matching those of miRNAs are likely to be true targets. In fact, much of the mRNA sequence is actually hidden, and only local single-stranded regions are accessible for binding to other RNAs, indicating that local target accessibility might lend specificity to the more promiscuous predictions of sequence matching. The 3′ untranslated regions (UTRs) of mRNAs form complex secondary and tertiary structures *in vivo* that depend on the intracellular environment. Unknown interactions within RNA transcripts or with proteins might result in further alterations of the 3′ UTR secondary structure (Box 1).

Table 1. Examples of functionally characterized miRNA targets

Organism	miRNA	Target	Refs
Caenorhabditis elegans	lin-4	*lin-14*	[3,4]
		lin-28	[70]
	let-7	*lin-41*	[74]
		daf-12	[75]
		lin-57	[76]
		Ras	[77]
		Nhr-25	[78]
	lsy-6	*cog-1*	[54]
	miR-273	*die-1*	[79]
	miR-61	*Vav-1*	[80]
Drosophila melanogaster	bantam	*Hid*	[6]
	miR-7	*Yan*	[69]
	miR-1	*Delta*	[34]
	miR-9a	*Sens*	[73]
	miR-278	*Expand*	[81]
Mus musculus	miR-196	*Hoxb8*	[82]
	miR-375	*myotrophin*	[83]
	miR-1	*Hand2*	[7]
		HDAC4	[38]
	miR-133	*SRF*	[38]
	miR-134	*Limk1*	[57]
Homo sapiens	let-7	*Ras*	[77]
	miR-15	*BCL2*	[84]
	miR-223	*NF1-A*	[59]
	miR-122a	*CAT-1*	[85]

miRNA functions: a developmental view

The dynamic nature of protein expression during cell-lineage decisions and subsequent morphogenetic events would be consistent with extensive regulation by miRNAs. In recent years, examples of tissue-specific roles of miRNAs during embryonic development have emerged and are reviewed here. Specifically, we focus on miRNA regulation of cardiac and skeletal muscle, neurons and hematopoietic lineages because accumulating evidence indicates pivotal functions for miRNAs in development of these cell types. The early role of miRNAs in stem-cell pluripotency and regeneration is also considered.

Global miRNA function in vertebrate development

One way to globally investigate the role of miRNAs in development is by examining mice that lack the essential miRNA-processing enzyme Dicer and, therefore, theoretically lack all miRNA function. Targeted deletion of Dicer in mice causes embryonic lethality before embryonic day (E) 7.5, suggesting an essential role for miRNAs in development. Moreover, Dicer-deficient embryonic stem (ES) cells are defective in differentiation both *in vitro* and *in vivo*, and do not form the three germ layers normally found in embryoid bodies derived from ES cells. However, Dicer might also be required for other small RNA-mediated pathways *in vivo*. Therefore, the role of miRNAs in early embryogenesis remains to be further characterized [43].

Tissue-specific inactivation of Dicer by using Cre-mediated recombination of loxP sites inserted around the gene encoding Dicer should reveal the requirement for miRNAs in specific tissues. For example, deletion of *Dicer* in discrete areas of the limb mesoderm in mice leads to severe growth defects in the limbs of mutant embryos, but not to defects in basic limb patterning or in tissue-specific differentiation [44]. Thus, miRNA function might be necessary for 'fine tuning' developmental events during the precise events of organogenesis. Consistent with this, disruption of *Dicer* in zebrafish reveals no apparent abnormality in axis- or pattern-formation events, and

Box 1. Role of mRNA target accessibility in miRNA–mRNA interactions

A prevailing view for miRNA target recognition is that the miRNA 5′ region, particularly nucleotides 2–7, known as the 'seed' region, is most important for miRNA–mRNA interactions via Watson–Crick base-pairing. Applying this criterion alone leads to prediction of many miRNA targets that cannot be validated *in vivo*, raising the likelihood that additional important rules control miRNA–mRNA interaction [87].

To determine the 'rules' governing miRNA-mRNA interactions, the properties of all validated targets at the protein level have been analyzed [7]. Most validated targets were discovered in worms or flies by genetic screens, and a handful were described in mammals. A common feature of all targets is that miRNAs preferentially target UTR sites that do not have a complex secondary structure and are located in accessible regions of the RNA, based on favorable thermodynamics [7] (Figure I). Since this model was proposed, several additional targets have been characterized *in vivo*, and almost all are consistent with the proposed accessibility criteria.

If miRNA target specificity is, in fact, determined by both sequence matching and target accessibility, this model has several implications. First, even targets with high sequence complementarity might not be the usual targets of a miRNA. As a corollary, however, these same sites might be rendered accessible under certain cellular conditions that would promote unfolding of stable secondary structures. This might introduce yet another layer of regulation of miRNA target selection that could be controlled by cellular events corresponding to various stresses, or regulated in a tissue-specific manner. miRNAs could function cooperatively to bind one accessible site, resulting in a melting effect on neighboring regions, thereby altering the secondary structure and enabling or inhibiting the binding of other miRNAs. As more is learned about target specificity, it will be interesting to test these hypotheses.

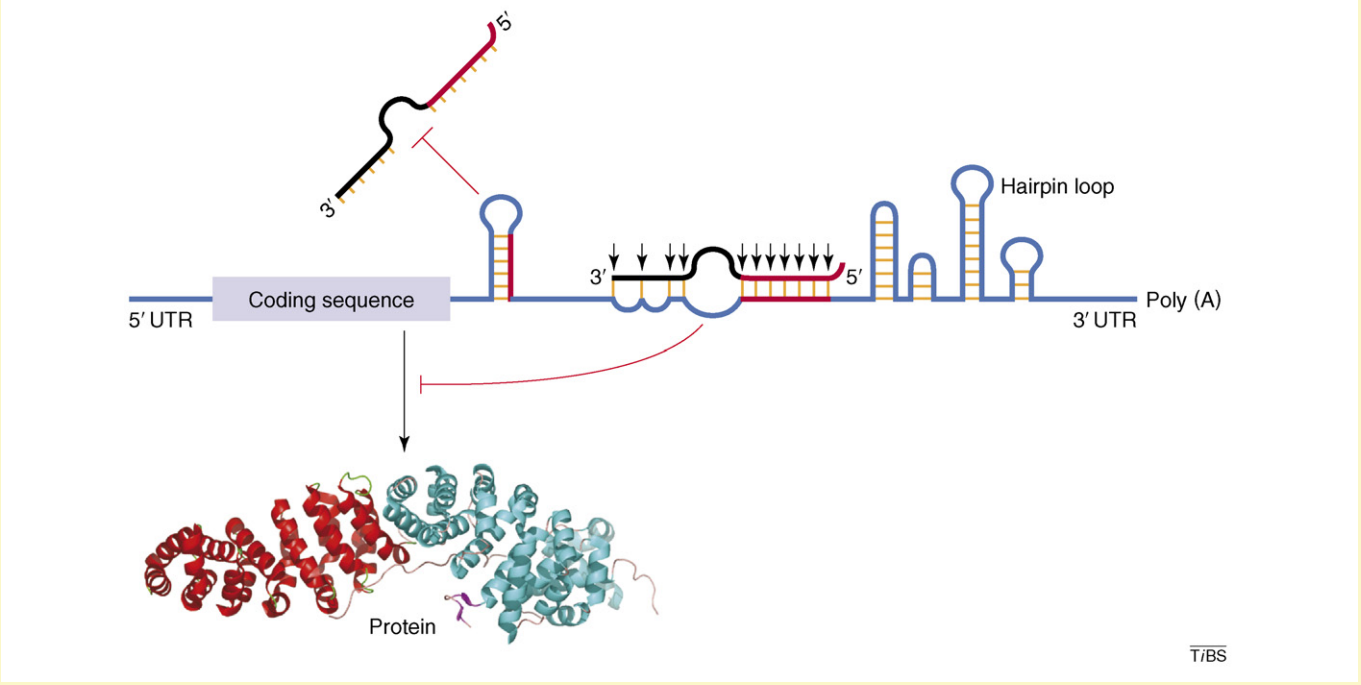

Figure I. Model of miRNA target accessibility. In addition to the well-described importance of sequence matching of particular residues (nt 2–7) in the 5′ end of miRNAs with mRNA targets, we propose that miRNAs can access their target sites only when they are physically accessible. Binding energy and secondary structure of the RNA, which itself could be regulated, might promote or inhibit miRNA–mRNA interactions. For example, despite a high degree of sequence matching to a region of the mRNA that forms a stem–loop or hairpin, a miRNA might not be able to access its binding site and, thus, would be unable to repress translation.

the major cell types and organs are present [45]. However, these mutants have severe defects in the form and function of many organs, including the brain and heart.

Cardiogenesis and myogenesis

The heart is the first organ to function in an embryo, and proper function of the cardiovascular system is crucial for embryonic survival during development. Cardiac morphogenesis involves an intricate coordination of multiple cell types during precise developmental windows [46].

As described, miR-1–1 and miR-1–2 are abundant in the developing heart [7]. Enrichment of miR-1–1 is initially observed in the atrial precursors before becoming ubiquitous in the heart, whereas a miR-1–2 enhancer is specific for the ventricles in the developing heart; therefore, miR-1–1 and miR-1–2 might have chamber-specific effects *in vivo* (Figure 3). Both are highly expressed in the cells of the cardiac outflow tract. Consistent with their regulation by myogenic transcription factors (SRF, MyoD and Mef2) [7,47,48], overexpression of miR-1 in the developing mouse heart results in early exit from the cell cycle and decreased ventricular proliferation and expansion. Using the criteria regarding mRNA target accessibility, Hand2, a bHLH transcription factor involved in ventricular cardiomyocyte expansion, has been identified as a target of miR-1 [7].

miR-1 and miR-133a are transcribed as a dicistronic primary message and, therefore, share common regulation. Both are transcriptionally regulated in cardiac muscle by SRF and in skeletal muscle by MyoD and Mef2 (Figure 3). miR-206, a close homolog of miR-1, is only expressed in skeletal muscle and is activated by MyoD. As is the case in cardiac muscle, miR-1 and miR-206 promote the differentiation of skeletal myoblasts in culture; miR-133a has the opposite effect, inhibiting differentiation and promoting myoblast proliferation. miR-1 family members, miR-206 and miR-133a, target a variety of factors that are

important for myogenesis, such as histone deacetylase 4 (HDAC4), SRF, Fstl1, Utrn, Pola1 and Cx43 [38,49–51].

Although no miRNA mutations have yet been described in mammals, disruption of the single fly ortholog of miR-1 results in uniform lethality at embryonic or larval stages with a frequent defect in maintaining muscle gene expression [33,34]. In a subset of flies lacking miR-1, a severe defect of cardiac progenitor-cell differentiation provides loss-of-function evidence that miR-1 participates in muscle differentiation events. Embryonic lethality is also observed by knocking down fly miR-1 activity by *O*-methyl-modified antisense oligonucleotides, although a milder miR-1 phenotype has also been described, suggesting a highly variable phenotype [52]. miR-1 in flies regulates the Notch signaling pathway by directly targeting mRNA of the Notch ligand, Delta [34], indicating that miR-1 functions to induce differentiated cardiac cells from an equivalency group of progenitor cells [53] (Figure 3).

Neurogenesis

miRNAs regulate key events during neurogenesis in multiple species, examples of which are described here. In *C. elegans*, two bilaterally symmetric gustatory neurons, ASE left (ASEL) and ASE right (ASER) exhibit left and right asymmetric molecular features with respect to one another. Auto-regulatory feedback loops involving distinct miRNAs control the cell-fate decision between the two asymmetric states. The transcription factors die-1 or cog-1 specify ASEL or ASER fates, respectively, by activating genes that distinguish ASEL from ASER, including the miRNAs lsy-6 and miR-273, which repress the ASER or ASEL fate, respectively. These cascades reinforce transcriptional programs leading to left–right asymmetry [54,55]. In zebrafish, maternal zygotic Dicer mutant embryos have severe defects in the formation of the neurocoel and the neural tube. These developmental defects are rescued by members of the miR-430 family, indicating that the latter are the major miRNAs involved in the neurogenic defects observed in the absence of miRNA biogenesis [56]. In mammals, miR-134 is specifically expressed in the rat dendritic spine in hippocampal neurons. It binds to 3′ UTR of Limk1 and represses local LimK1 translation, which results in inhibition of dendritic-spine growth. Stimuli such as brain-derived neurotrophic factor can relieve this suppression. This finding revealed that miRNAs can be important regulators of neuronal structure and plasticity and can somehow respond to external signals [57].

Hematopoiesis

During embryogenesis, active hematopoiesis begins in the yolk sac and then moves to the liver, and, in later stages, to the bone marrow. In normal adults, hematopoiesis occurs in marrow and lymphatic tissues. The mechanisms governing changes in the site of hematopoiesis involve sequential cell-fate diversification and cell differentiation. miR-181 is highly expressed in B-lymphoid cells of the bone marrow and ectopic expression of miR-181 in progenitor cells increases the fraction of B-lineage cells *in vitro* and in adult mice, although the underlying mechanism is poorly understood [58]. Similarly, miR-223 and two transcription factors, nuclear factor 1-A (NF1-A) and CCAAT enhancer-binding protein α (C/EBPα), are involved in granulopoiesis and function in a negative feedback loop to regulate granulocytic differentiation [59].

Stem cells, regeneration and homeostasis

Most somatic cells in *C. elegans* and *Drosophila* are post-mitotic and lack adult somatic stem cells; by contrast, germline cells maintain pluripotential ability to renew themselves indefinitely. Dicer and Drosha loss-of-function mutants in *C. elegans* indicate a role for miRNAs in germline stem cells [60,61]. Similarly, Dicer-1 mutants in *D. melanogaster* show a stem-cell self-renewal defect in germline stem cells. The underlying mechanism seems to be a block in the G1/S transition phase [62].

The flatworm *Schmidtea mediterranea* is an established model for regeneration mediated by stem cells that have roles in tissue maintenance and regeneration. The Argonaute-like proteins smedwi-1 and smedwi-2 are required for adult somatic stem-cell functions in tissue regeneration and homeostasis. This indicates that pathways involving small RNAs – presumably, miRNAs – are important in the regenerative role of stem cells [63].

As in other tissues, a distinct set of miRNAs is specifically expressed in pluripotent ES cells but not in differentiated embryoid bodies, suggesting a role in stem-cell self-renewal [64]. Dicer1-null ES cells, which lack mature miRNAs, fail to differentiate into the three germ layers [65,66]. In Dicer1-null mouse embryos, the pool of pluripotent stem cells in the inner-cell mass of the blastocyst is diminished [43]. It will be interesting to determine which miRNAs regulate self-renewal versus differentiation and the targets through which they execute these decisions.

Modes of miRNA-mediated regulation

Many paradigms have emerged over the past decade that govern the precise and coordinated interpretation of genetic information during embryonic development. As a recently recognized part of that regulation, miRNA-mediated events seem to ensure the preciseness and fidelity of dynamic and spatially restricted gene expression during development. Some aspects of miRNA-mediated regulatory circuits that might control developmental events are considered here.

Regulation of expression thresholds

The importance of gene dosage is well established, especially in developmental processes such as patterning. Multiple lines of evidence indicate that miRNAs are involved in fine-tuning biological processes by titrating precise dosages of regulatory proteins. For example, the concentration of lin-41 might be critical for the larval stage 4 (L4)-to-adult transition in worms, and might be suppressed by let-7 to below threshold levels [67]. Similarly, cell death can be sensitive to the dosage of bantam [6]. In flies, the number of cardiac progenitor cells seems to be sensitive to the dosage of miR-1, which regulates the threshold of crucial signaling and transcriptional events, including those involving the Notch pathway. The net effect might result in abnormal cardiac progenitor cell-fate diversification, manifested as an alteration in the pool of cardiac progenitors [34].

In some instances, upon reaching a threshold, the action of miRNAs can have a switch-like effect, such as lin-4 repression of lin-14 [3,4]. Interestingly, although let-7 is expressed as early as the L2 stage, it doesn't elicit its blockage effect on lin-41 until the L4 stage when let-7 is highly expressed [67,68].

Feedback regulation

A key feature of embryogenesis is that cell-fate specification and differentiation progress in a unidirectional manner during development. At the molecular level, differentiation involves the conversion of transient signals into stable circuits of cellular identity. Commonly, the differentiation program of a given cell lineage is determined and stabilized by feedback loops, as described in mammalian muscle, for the miR-1 and miR-133 and SRF–Mef2–MyoD circuitry (Figure 3).

Late in development, miRNAs are important in cell-lineage diversification (as mentioned). Not surprisingly, miRNA-mediated regulation frequently contains auto-regulatory feedback loops. In worms, miRNAs function in a double-negative feedback loop to control a neuronal cell-fate decision [54,55]. In *Drosophila* eye development, undifferentiated retinal progenitor cells express the transcription factor Yan, which inhibits the transcription of miR-7. The activation of photoreceptor differentiation signals downregulates Yan activity, relieving its repression on miR-7 expression, which subsequently binds to the 3′ UTR of Yan to block its translation [69]. We predict that miRNA-mediated feedback loops will prove to be important in the reinforcement of multiple aspects of tissue differentiation and organogenesis.

Individual versus cooperative target regulation

It is interesting to consider whether individual miRNAs function independently on specific targets or if they can also function in a combinatorial manner, similar to the combinatorial interactions of transcription factors used to exponentially increase the number and specificity of targets. Current evidence supports both scenarios. An example of the effects of single miRNAs can be found in the regulation of lin-28 mRNA by lin-4 [70]. By contrast, many miRNA clusters reside in corresponding introns of paralogous genes. These polycistronic miRNAs can usually be classified into the same miRNA family based on sequence similarity in the miRNA 5′ region, suggesting that they might cooperatively regulate common sets of targets or molecular events [32]. For example, let-7 regulates a heterochronic gene target, *hbl-1*, during the L2-to-L3 transition in worm. The let-7 family includes miR-48, mir-84, mir-241 and let-7, all of which function cooperatively to repress the heterochronic gene, *hbl-1*, through direct interaction with the 3′ UTR [68]. In addition to coordinately regulating a single mRNA, multiple members of a miRNA family can regulate sequential events, as observed for miR-430 during brain morphogenesis in zebrafish [56].

Reinforcement of transcriptional silencing

In loss-of-function and gain-of-function studies, mutants of several miRNAs (e.g. lin-4, let-7, miR-1 and bantam) display specific phenotypes, indicating that miRNAs have important roles in the expressed tissue or organ [3,4,6,7]. The mRNA targets are often co-expressed with the miRNAs, which regulate dosages of proteins that have important functions in the given tissue. By contrast, miR-196 functions in a fail-safe mechanism to silence inappropriate Hox activity during limb development [71]. Recently, it has been proposed, based on bioinformatics analyses, that miRNAs and their targets are often expressed in mutually exclusive expression domains [72]. Therefore, another task of miRNA might be to prevent inappropriate activity of genes in domains in which they are already repressed transcriptionally. As an example, a crucial role for miR-9a in ensuring precise sense-organ specification through repression of the transcription factor Senseless (sens) was reported recently [73]. *Drosophila* miR-9a is expressed in the cells surrounding the sensory-organ precursors (SOP) but not in the SOP. By contrast, sens is expressed at high levels in the SOP cells and at low levels in the surrounding cells. It is suggested that miR-9a downregulates *sens* expression, and thus reinforces the distinct expression pattern of sens to ensure proper organ specification.

Concluding remarks

The past several years have witnessed tremendous progress in our understanding of miRNAs. Many have important roles in a broad range of developmental processes, but the number of miRNAs and their roles are still emerging. However, it is becoming clear that miRNAs are integrally involved in the complex regulatory networks that govern the developmental, homeostatic and physiological processes of most organisms. Because this field is still in its infancy, several important questions remain. The most fundamental challenge is to define the rules of miRNA target recognition. This is essential because the biological role of individual miRNAs will be dictated by the spectrum of mRNAs that they regulate. As additional targets are validated, it might be possible to establish commonalities that enable more precise prediction of miRNA–mRNA interaction. Beyond identification of targets, it will be important to define the endogenous roles of miRNAs *in vivo*. Currently, the functions of specific mammalian miRNAs are postulated either from gain-of-function studies *in vivo* or in cell-culture systems. Loss-of-function of a specific miRNA has not yet been reported in mammals, but targeted deletion of individual miRNAs in mice will undoubtedly provide important insight into normal miRNA function. Finally, understanding how miRNAs are processed and how they are integrated into the complex regulatory networks that govern the developmental, homeostatic and physiological processes of organisms will be crucial. In particular, given the ability of miRNAs to regulate multiple genes, it will be interesting to investigate whether they function through many of the same paradigms as transcription factors, such as combinatorial regulation and regulation of whole genetic programs. If the breadth of regulation by miRNAs is as predicted, it will be yet another example of the elegant use of simple tools to solve complex problems in nature.

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