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Adult-specific functions of animal microRNAs

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Abstract

microRNAs (miRNAs) are ~22 nucleotide (nt) RNAs that coordinate vast regulatory networks in animals, and thereby influence myriad processes. This review examines evidence that miRNAs play continuous roles in adults, in ways that are separable from developmental control. Adult-specific activities for miRNAs have been described in a variety of stem cell populations, in the context of neural function and cardiovascular biology, in metabolism and physiology, and during cancer. In addition to reviewing recent results, we also discuss methods for studying miRNA activities specifically in adults and evaluate their relative strengths and weaknesses. A fuller understanding of continuous functions of miRNAs in adults has bearing on efforts and opportunities to manipulate miRNAs for therapeutic purposes.

Introduction

Developmental studies during the 1990s revealed the first endogenous ~22-nt regulatory RNAs in *Caenorhabditis elegans*^{1, 2} and demonstrated that diverse 7-nt 3' UTR motifs mediated critical modes of post-transcriptional repression in *Drosophila melanogaster*^{3, 4}. In the past decade, these RNAs and motifs were recognized as central components of the miRNA pathway^{5–8}, in which hairpin transcripts are processed into short RNAs that associate with Argonaute proteins, and guide them to target mRNAs for degradation and/or translational inhibition. Animal genomes typically harbor hundreds to >1000 miRNA loci, which are collectively inferred to target the majority of transcripts in most studied invertebrate and vertebrate species^{9, 10}. Consequently, nearly all developmental, physiological, and disease-related processes appear to be regulated by miRNAs, at least to some degree¹¹.

In this review, we focus on adult roles of miRNAs. Many compelling developmental phenotypes caused by miRNA dysfunction are also manifest in adults, but here we are specifically interested in cases where miRNAs play continuous and active roles during adult stages. These cases have particular bearing on the extent to which miRNA-related syndromes might be amenable to therapies involving re-supply or inhibition of miRNAs, as such treatments may not be effective if the root miRNA involvement occurred during development. Equally important are demonstrations that adult-specific manipulation of

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miRNAs can reverse pre-existing conditions. Excitingly, such data has indeed begun to emerge. We refer readers to other reviews for background information on miRNA biogenesis and function^{12, 13} and topic-specific discussions that include developmental and/or cultured cell studies^{14–16}.

There are technical challenges to separate adult phenotypes from phenotypes that have a developmental basis, but many strategies are now available. miRNA genes are amenable to loss- and gain-of-function methods used to manipulate protein-coding genes¹⁷. Also, mutations in core miRNA biogenesis factors, such as Dicer, are widely used to assess global miRNA depletion (with the caveats that many non-canonical miRNA biogenesis pathways exist, and several core miRNA biogenesis factors have non-miRNA substrates¹²). These strategies can be aimed at adult-specific functions using inducible or tissue-specific systems (Box 1 and Figure 1). In addition, miRNAs can be inhibited using antagomirs and sponges (Box 1 and Figure 1). We highlight major current methodologies, discuss issues in interpreting results from different strategies (Box 2), and emphasize that multifaceted approaches are necessary for confident conclusions.

Box 1

Strategies for manipulating miRNA activity in vivo

miRNAs are generally amenable to loss- and gain-of-function methods used to manipulate protein-coding genes. In addition to manipulations of individual miRNAs, mutants of core miRNA biogenesis factors frequently provide a proxy for assessing potential influence of miRNAs. Dicer has genetically been the most well-studied miRNA factor, but the existence of alternative miRNA pathways¹² suggests that multiple miRNA components should be studied in parallel. In addition, the study of miRNAs has necessitated the development of novel methodologies, including antagomirs and sponges¹⁷. Collectively, these techniques have been used to demonstrate “adult” miRNA functions across diverse tissues.

Mutations and transgenes

Deletions of ~100 *C. elegans* miRNAs have been made¹⁸. Although few exhibit developmental or physiological defects on their own, many cause phenotypes upon genetic sensitization¹⁶⁴. Phenotypes were reported for a few dozen *D. melanogaster* and mouse miRNA deletions^{15, 162, 167}, and two large-scale efforts have targeted hundreds of miRNA loci in mouse ES cells^{168, 169}. These mutants were mostly assayed as constitutive deletions, but genetic evidence for active roles of miRNAs in adult stages requires conditional knockout. *Drosophila* studies use Flp recombinase to mediate mitotic recombination between FRT target sites on homologous chromosomes¹⁷⁰ (Figure 1A). Adult-specific clones can be generated using heat shock-inducible Flp; however, cell division is required for this homologous recombination based method. In mice, conditional knockouts use Cre recombinase to excise a gene region flanked by loxP sites (Figure 1B). Although some Cre drivers are restricted to postnatal or adult stages, temporal control is usually achieved using Cre fused to the estrogen receptor (Cre-ER). Upon administration of tamoxifen, Cre-ER translocates to the nucleus and becomes active.

As some processes are affected by estrogen analogs, tetracycline (Tet)-controlled systems offer an alternative. In the Tet-off system, administration of Tet or its analog doxycycline (Dox) prevents tetracycline-controlled transactivator protein (tTA) from activating target genes linked to tTA binding sites; thus Dox turns the system off. The Tet-on system utilizes reverse tTA (rtTA) protein, which only recognizes DNA in the liganded state. These systems allow temporal control of Cre. In addition, they can be used to directly control individual miRNAs, such as to show that an adult phenotype requires ongoing miRNA synthesis. Most fly transgenes are activated using the Gal4-upstream activation sequence (Gal4-UAS) system, which can be temporally controlled using heat-shock-Gal4, a temperature-sensitive repressor of Gal4 (Gal80^{ts}), or progesterone-regulated Gal4.

Finally, a temperature-sensitive allele of the miRNA biogenesis factor *pasha* was recently identified in *C. elegans*¹⁰⁷ (Figure 1C). This unique reagent permits temporal blockade of miRNA biogenesis, including during the strictly post-developmental and post-mitotic context of adult nematodes. Such a valuable reagent, which can be used in a reversible fashion, may encourage engineering temperature-sensitive versions of core miRNA factors in other species.

Antagomirs and sponges

Due to the time and expense involved in generating knockout strains, efforts have been devoted to simpler alternatives for miRNA loss-of-function. Two major strategies involve antagomirs and sponges (Figure 1D), which titrate miRNAs from their targets and/or potentially induce their turnover. In theory, delivery of antagomirs and sponges in adult stages can provide evidence for continuous post-developmental requirements for miRNAs.

Antagomirs are antisense miRNA sequences built on a modified backbone involving locked nucleic acids (LNAs), which increase duplex stability and resist degradation¹⁷¹. These can be transfected into cultured cells, but delivery *in vivo* requires conjugated cholesterol to enhance cellular uptake¹⁷². “Tiny LNAs” are complementary to ~8nt miRNA seed sequences and can block entire miRNA families¹⁷³. Sponges are decoy transcripts bearing binding sites to a miRNA of choice¹⁷⁴, and can be expressed by transfection, integrated transgene, or viral transduction. These utilize a multimer (4–20 copies) of bulged binding sites to “distract” miRNAs from their endogenous targets¹⁷⁵. Since many miRNAs are expressed at high levels, high sponge levels are required. Recently, “tough decoys” bearing two target sites in a structured hairpin were reported to have favorable stability and efficacy¹⁷⁶, spurring generation of large-scale resources for *in vivo* expression of tough decoys^{177, 178}. The capacity to express sponges transgenically is advantageous as a sustainable and/or regulatable source of the decoy.

Box 2

Phenotypic discrepancies between antagomirs and knockouts

miRNAs have inherently limited sequence ‘real-estate’ for inhibitor design. An early study used multiple antagomirs against non-overlapping regions of the primary miRNA

hairpin, to inhibit different steps of miRNA biogenesis¹⁷⁹. Independent miR-375 antagomirs induced defects in pancreatic islets, where miR-375 is normally expressed. However, since then, almost all antagomir studies have used a single species, perfectly complementary to the miRNA. Depletion of the target miRNA can be demonstrated by Northern or qPCR analysis, and functional target derepression can be seen on the transcriptome scale. However, it is difficult to determine whether off-target effects have occurred. With RNAi techniques, on-target silencing can easily be assayed, but the widespread appreciation of off-target effects has made it standard practice to utilize multiple siRNAs or shRNAs to replicate phenotypes.

Reason to be cautious in interpreting antagomir phenotypes has come from *Drosophila*. Initial studies described a cornucopia of apparently specific defects induced by different miRNA antagomirs¹⁸⁰. Antagomir phenotypes discriminated amongst members of some miRNA families, and antagomir-mediated phenotypes could be rescued by miRNA overexpression. Given this, it is surprising that most reported *Drosophila* antagomir phenotypes were not recapitulated by deletion alleles generated subsequently.

Antagomirs have overall been little-used in invertebrates, but their use has increased dramatically in mammalian systems. It is reassuring that some concordant results have emerged from antagomirs and knockouts. For example, anti-miR-122¹⁷² and a *mir-122* knockout^{136, 137} both reduced serum cholesterol, due to downregulation of cholesterol biosynthesis genes. However, several antagomir-mediated phenotypes have not been recapitulated by mouse knockouts. For example, miR-21 antagomirs prevented hypertrophy and fibrosis in the failing heart, and thereby improved cardiac function¹⁸¹. However, *mir-21* knockouts did not reveal alteration of cardiac remodeling following stress, nor did application of functional miR-21 tiny LNAs¹⁸². Studies of miR-143/145 antagomirs indicated critical roles in smooth muscle development¹⁸³, but *mir-143/145* knockouts showed largely normal smooth muscle differentiation^{184, 185}. However, the *mir-143/145* deletion did exhibit defects in phenotypic switching of smooth muscle cells in vascular remodeling after injury. Finally, mammalian *mir-34* is transcriptionally activated by p53, and antagomir studies provided evidence that inhibition of the miR-34 family interfered with the p53 response^{125–127, 186–188}. However, recent thorough analysis of a triple *mir-34a/b/c* mouse knockout failed to reveal phenotypic defects, even in cancer models¹³³.

The bases for discrepancies between the antagomir and knockout studies remain to be clarified. Notably, “scrambled” controls do not exclude the possibility that an antagomir has some specific off-target effect, and rescue of antagomir phenotypes by delivering cognate miRNA mimics does not formally distinguish titration of antagomirs from on-target or off-target species. A clear test would be to repeat antagomir studies in a miRNA knockout background. One might expect that antagomirs should not induce further phenotypes without their targets. If that is the case, germline miRNA knockouts might be associated with developmental compensation, whereas acute miRNA inhibition by an antagomir might cause a functional imbalance yielding an adult phenotype. On the other hand, if antagomirs recapitulate their phenotypes in knockouts, off-target effects should be considered. Although there is usually limited enthusiasm for performing experiments

for which the best outcome would be a negative result, these are critical tests to perform if miRNAs are to eventually be used as therapeutics.

Given that many miRNA mutants lack obvious phenotypes^{18, 19}, the generation of robust adult-specific phenotypes is not trivial. Notable circumstances and processes that exhibit distinctive requirements for miRNAs include stem cells (which must generate precise cell lineages throughout life), neurons (which are long-lived and have exceptional morphology), metabolic organs (which must maintain homeostasis from ever-changing nutritional inputs), and during aging (which is perhaps the ultimate adult-specific process). Finally, there is increasing evidence that some cancers are actively driven by gain or loss of specific miRNAs. Such findings provide a rationale and proof-of-principle that stem cell alterations, neurological disorders, metabolic imbalances, longevity and cancers may eventually be amenable to miRNA-based therapies.

Adult stem cell maintenance and differentiation

Proper self-renewal, proliferation and differentiation of stem cells are important for diverse aspects of adult physiology (Figure 2A), and abnormal stem cell activities underlie many diseases and cancers. Blocking the miRNA biogenesis pathway in adults through ubiquitous knockout of *Dicer* using an inducible Cre recombinase (*Rosa26-CreERT2*) resulted in defects in several tissues. The mice rapidly developed intestinal deterioration and succumbed within 10 days, with additional defects in bone marrow, spleen and thymus²⁰. Such phenotypes implied continuous requirements for the miRNA pathway in multiple tissues that undergo turnover and are renewed by stem cells. Here, we discuss studies that illuminate roles for miRNA biogenesis and individual miRNAs in a selection of adult stem cell settings.

Germline stem cells

Requirements for miRNAs in adult stem cells were first shown during self-renewal of *D. melanogaster* ovarian germline stem cells (GSCs). Adult clones bearing temporally-controlled knockout of *dicer-1* (see Box 1 for experimental details) showed reduced production of germline cysts²¹, and subsequent studies demonstrated that multiple components of the miRNA pathway are required for GSC self-renewal^{22–25}. Mutant clones of the bantam miRNA exhibit progressive GSC loss, indicating its relevance to GSC dynamics²⁶. More recently, analysis of *drosha*, *pasha*, *dicer-1* and *ago1* mutant clones revealed a common requirement for the miRNA pathway in oocyte determination and germline cell division²⁷.

As sperm are produced throughout the life of adult mammalian males, one might wonder if miRNAs exhibit ongoing requirements in testes. Recently, *Dicer* was removed postnatally in mice using the male germ cell-specific deleter *Stra8-Cre*, whose activity initiates at 3 days postnatal (P3)²⁸. Sperm numbers decreased by 5 weeks and were nearly absent by 8 weeks, with the remaining mutant sperm exhibiting defective morphology. These mice were infertile and accumulated gametes with leptotene and zygotene chromosomes, demonstrating that *Dicer* is essential for adult male germ cells to progress beyond early

meiotic stages. Interestingly, molecular defects evident at P18 included preferential upregulation of X- and Y-linked genes, which was interpreted as a failure of meiotic sex chromosome inactivation²⁸.

Neural stem cells and neurogenesis

Two major niches for neural stem cells in the adult brain are the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus, and ongoing adult neurogenesis contributes to olfaction, learning and memory²⁹. In the adult SVZ neural stem cell (NSC) niche in mice, the CNS-specific miRNA miR-124 controls temporal progression of neurogenesis through repression of the SRY-box transcription factor SOX9³⁰, which directs NSC self-renewal and multipotency³¹ (Figure 3A). This miRNA is not expressed in NSCs, but is activated as they differentiate into transit-amplifying cells and is maintained in neuroblasts and newborn neurons³². Sustained inhibition of miR-124 in the adult SVZ using a lentiviral sponge showed its requirement for neurogenesis and for preventing gliogenesis. A critical role for miR-124 in neurogenesis is consistent with the finding that this miRNA can promote fibroblasts to adopt an induced neuron fate³³. Curiously, overexpression of miR-124 in the SVZ is not compatible with sustained neurogenesis, but rather supports only a single wave of neurogenesis. Ectopic expression of miR-124 in adult NSCs leads to their premature differentiation and thus depletes SVZ stem cells³².

Repression of the transcription factor PAX6 by miR-7a underlies the regionalization of the SVZ stem cell pool³⁴. PAX6 is a molecular determinant of newborn dopaminergic neurons in the olfactory bulb, which originate from the dorsal SVZ. Ablation of the miR-7a site in *Pax6* mRNA 3' UTR, or electroporation of a miR-7a sponge, relieved miR-7a repression and led to PAX6 expression in the ventral SVZ and thus dopaminergic neuron production from ventral stem cells. Finally, in the hippocampus, the expression of miR-137 and miR-184 is controlled by DNA methylation and these miRNAs regulate the balance between proliferation and differentiation of adult hippocampal NSCs^{35, 36} (Figure 2B). Perhaps paradoxically, while miR-137 expression is significantly increased during normal NSC differentiation, precocious expression of this miRNA in NSCs promotes proliferation whereas inhibition enhances differentiation, indicating the requirement of proper temporal control of miR-137 for hippocampal neurogenesis³⁶.

Hematopoietic stem cells (HSCs)

The rapid turnover, self-renewal and hierarchical differentiation of HSCs are regulated by complex mechanisms to maintain homeostasis of the blood system. miRNA profiling in hematopoietic lineage cells has revealed distinct miRNA signatures for stem, progenitor and differentiated cell populations, suggesting that miRNAs are active regulators and effectors during hematopoiesis^{37, 38}.

The *Mx1-Cre* deleter is strongly induced in liver and lymphocytes upon treatment with interferon or by injection of polyinosinic-polycytidylic acid (pI:pC), and this system was used to excise *Dicer* in adult mice³⁹. Bone marrow from the mutant mice was tested under competitive conditions in a repopulation assay in lethally irradiated recipient mice. Analysis

of T cell, B cell and myeloid lineages in the recipients showed that loss of *Dicer* in HSCs strongly reduced their ability to contribute to these compartments due to HSC apoptosis. Follow-up analysis showed that miR-125a is highly expressed in HSCs and promotes their survival³⁹.

Many knockouts of hematopoietic miRNA loci have been characterized, as reviewed recently⁴⁰. With the exception of a compound *mir-17-92* cluster knockout that removed 6 miRNAs⁴¹, all the miRNA knockout mice develop normally and are fertile. Therefore, they have mostly been studied in the context of germline inheritance of the mutations. Nevertheless, individual miRNAs clearly have diverse roles in maintaining the appropriate balance of differentiated blood cell types. For example, *mir-223* mutants exhibit a hyperactive immune response caused by increased proliferation of granulocyte progenitors mediated by derepression of the transcription factor encoded by *Mef2c*⁴² (Figure 2B). In the lymphoid lineage, miR-150 controls B cell differentiation through regulation of the c-MYB transcription factor⁴³. Analyses of stress conditions have also been informative. For example, deletions of *mir-144/-451* or *mir-451* alone caused mild anemia, but led to a striking inability to regenerate red blood cells following oxidative stress⁴⁴⁻⁴⁶, in part due to deregulation of 14-3-3 epsilon, which is a regulator of cytokine signaling.

Given the complexity of the hematopoiesis, a special feature of miRNAs in the HSC lineage is their involvement in the regulation of cell fate specification during steps of hematopoietic stem and progenitor cell differentiation. In addition to its function in lymphocyte development, miR-150 also regulates lineage commitment during megakaryocyte-erythrocyte progenitor differentiation; high levels of miR-150 shift the balance towards megakaryocytes at the expense of erythrocytes⁴⁷. This function of miR-150 is also mediated by c-MYB. These studies provide a flavor of the diverse roles of miRNAs in maintaining appropriate homeostasis of the blood system.

Satellite cells and skeletal muscle

Satellite cells (SC) comprise the major stem cell pool in adult skeletal muscles, and mediate muscle regeneration in response to acute injuries and muscular dystrophies⁴⁸. SC stemness and proliferation of SC-derived myogenic progenitors are marked by expression of the paired box family transcription factors, PAX7 and PAX3. Both are targeted by multiple miRNAs to promote myogenic differentiation^{49, 50}. The related myogenic miRNAs miR-1 and miR-206 are induced during SC differentiation and repress *Pax7* (Figure 2B). During muscle regeneration, when SCs proliferate actively, these miRNAs are sharply downregulated⁵⁰. Application of antagomirs against these miRNAs increases SC proliferation in normal adult skeletal muscle; and preventing the interaction of miR-1 or miR-206 with *Pax7* impairs myoblast differentiation *in vitro*. Moreover, *mir-206* knockout mice exhibit delayed muscle regeneration after injury, and exacerbated the dystrophic phenotype of a mouse model of Duchenne muscular dystrophy⁵¹. Similar regulatory interactions between miR-27b and PAX3 have been observed in some adult SCs⁴⁹. Transforming growth factor β (TGF- β) and bone morphogenetic protein (BMP) signaling is also required to maintain muscle stem cells, and is downregulated during myogenic differentiation. A regulatory function of miR-26a, similar to that of miR-1/-206, was

recently shown to control the levels of TGF- β and BMP signaling during muscle differentiation and regeneration via repression of the Smad transcription factors⁵².

Muscle stem cell quiescence is critical since aberrant activation of SCs depletes the stem cell pool⁵³. Loss of miRNA biogenesis enables SCs to spontaneously enter the cell cycle⁵⁴. Two recent studies showed that miR-489 and miR-31 maintain SC quiescence through distinct mechanisms. miR-489 is enriched in quiescent SCs and quickly downregulated upon SC activation. Ectopic expression of miR-489 maintains SC quiescence and impairs muscle regeneration after injury, and ablation of miR-489 using an antagomir caused spontaneous SC activation in uninjured skeletal muscles. A relevant target of miR-489 is the oncogene *Dek*, which promotes myogenic progenitor proliferation⁵⁴. In contrast, miR-31 levels are not dynamic in the SC lineage, but this miRNA forms mRNP granules that sequester and silence the myogenic determinant *Myf5* mRNA in quiescent SCs. During activation, the granules disassemble to allow rapid translation of MYF5, which promotes myogenesis⁵⁵.

Skin biology

The skin is continually replenished via stem cells in the innermost basal layer. An advantage to studying the skin is that it can be directly accessed *in situ*. Therefore, temporally-controlled knockouts can be generated simply by topical administration of tamoxifen to mice carrying *K14-CreER*, which is active in the basal epidermal layer. Using this approach, deletion of *Dicer* from adult skin caused epidermal thickening and presence of ectopic suprabasal cells that express the stem cell marker p63, which is a target of the abundant epidermal miRNA miR-203⁵⁶.

Hair follicles undergo cycles of growth (anagen), regression (catagen) and rest (telogen), which can be experimentally induced by hair plucking. A doxycycline (Dox)-inducible Cre (*Krt5-rtTA/tetO-Cre*, see Box 1 for details on this expression system) that is active throughout the basal epidermis and in hair follicle cells of mice was used to delete *Dicer* and *Drosha* at different time points during the hair follicle cycle⁵⁷. In contrast to the embryonic requirement of miRNAs in establishing the stem cell compartment, adult-induced loss of miRNAs in telogen did not affect resting hair follicles. However, after hair plucking, mutant follicles failed to grow normally, accompanied by apoptosis of the transient amplifying follicular matrix cells and degradation of hair follicles⁵⁷. This suggested a requirement of the miRNA pathway specifically in anagen phase in adult skin, potentially via the abundant epithelial miRNA miR-205.

Central nervous system

Neurons are very long-lived and exhibit extreme morphologies, which are properties that suggest that they may have special requirements for post-transcriptional regulatory mechanisms. Many miRNAs and some miRNA pathway factors are enriched in synapses or dendrites relative to cell bodies⁵⁸, supporting the idea of local translational control by miRNAs. Moreover, miRNA expression is often regulated in an activity-dependent fashion. Although miRNAs are broadly considered to be fairly stable, studies in the adult retina of mice revealed several miRNAs that are subject to rapid post-transcriptional downregulation

following dark adaptation⁵⁹. Altogether, the activity of the adult nervous system is clearly regulated by miRNAs, and miRNAs are reciprocally regulated by neural activity.

Neuronal survival

Studies in cultured neurons and embryonic nervous system provide broad evidence for neural functions of miRNAs^{58, 60}, but it is not trivial to analyze this specifically in adult brains. Conditional knockout of *Dicer* in mouse embryonic brain revealed strong dependence of neuronal survival and differentiation on miRNAs^{61, 62}. Therefore, potential developmental bases of conditional knockout phenotypes studied in adults, or of viable miRNA knockouts, must be considered. In addition, substantial neural development occurs during postnatal, pre-puberty stages, and few studies have deleted miRNA components in the adult nervous system using temporally controlled systems. However, as a start, some studies have utilized Cre drivers that are active in post-mitotic neurons and/or that have a post-natal onset of activity. For example, excision of *Dicer* using *Pcp2-Cre*, which is specifically activated in differentiated Purkinje neurons ~two weeks after mouse birth, did not cause obvious defects up to 10 weeks of age. However, from 13 weeks onward, Purkinje cell death, cerebellar degeneration, and ataxia were evident⁶³. *Dicer* is also important in non-neuronal cells of the brain; postnatal deletion in astroglial cells, as driven by *GFAP-Cre*, resulted in neuronal degeneration, neuronal dysfunction, and seizures⁶⁴.

Learning and memory

One analysis of a *Dicer* conditional knockout in the fully-formed adult mouse brain (at 8–10 weeks) used the inducible *CamKII-CreER2* deleter, which is active in the forebrain⁶⁵. A surprise was that these mice exhibited enhanced learning and memory and increased synaptic transmission in the hippocampus; these phenotypes were observed up to 3 months and then neurodegeneration ensued. Although permanent loss of the miRNA pathway is obviously detrimental, these experiments raise the provocative question of whether reversible modulation of the miRNA pathway or of individual miRNAs can promote learning. The transcription factor CREB is a master regulator of synaptic plasticity and cognitive functions, and several miRNAs have been linked to the CREB axis. For example, mutation of the deacetylase SIRT1 downregulates CREB expression and impairs long term potentiation (LTP), as a result of increased miR-134. In particular, delivery of miR-134 antagomirs suppressed contextual fear memory defects in *SIRT1* mutant mice⁶⁶. In addition, adult silencing of miR-134 conferred neuroprotection during epileptic seizure, which is another condition that upregulates miR-134⁶⁷. There is also substantial evidence connecting the *mir-132/212* cluster, a transcriptional target of CREB, to diverse aspects of dendritic plasticity, learning and memory⁶⁸. Finally, a recent study uncovered details of a remarkably linear pathway involving transcriptional regulation of miR-124 and its downstream target, which altogether control LTP, memory and behavior⁶⁹ (Figure 3B).

Innate and adaptive behaviours

In addition to cognitive behaviors, miRNAs influence innate behaviors. Circadian rhythm provides a well-studied example. In *D. melanogaster*, postmitotic depletion of miRNA biogenesis in circadian pacemaker neurons dampened the amplitude of locomotor

rhythmicity⁷⁰. Recent studies provide more specific evidence of miRNA-mediated regulation of the circadian clock. For example, *mir-279* mutant flies exhibit circadian arrhythmia through altered JAK/STAT signaling⁷¹. Knockdown of the miRNA co-effector GW182 in circadian neurons altered circadian activity patterns in ways that closely resemble mutants of Pdf (a neuropeptide that regulates circadian rhythms) and the Pdf receptor⁷². Interestingly, heterozygosity of *dunce*, a negative regulator of Pdfr/cAMP signaling, significantly rescued the GW182 knockdown phenotypes, suggesting that it may be a critical target under miRNA regulation⁷². In mammals, *mir-132* and *mir-219* were the first miRNAs to be functionally connected to circadian rhythms, as shown by intraventricular infusion of antagomirs against them⁷³. *miR-219* is a target of the CLOCK/BMAL1 transcriptional complex and regulates period length, whereas *miR-132* is induced by light cues and modulates light-induced clock resetting. More recently, adult-specific expression of a Tet-inducible *mir-132* transgene, controlled by the *Camk2a-tTA* activator transgene (see Box 1 for details on the tTA system), dampened circadian cycling of Period protein and attenuated light-induced clock resetting⁷⁴. This was linked to direct repression of multiple chromatin remodeling and translational control factors by *miR-132*.

miRNAs are also involved in diverse adaptive behaviors, including drug addiction^{58, 60}. In the suprachiasmatic nucleus and striatum, *miR-9* is induced by alcohol and selectively regulates large-conductance, calcium- and voltage-activated (BK) potassium channel variants, thereby dampening response to alcohol and inducing tolerance⁷⁵. Cocaine intake is also modulated by striatal miRNAs. *miR-212*, a member of the CREB-responsive *mir-132/212* cluster, is upregulated during extended cocaine intake. This miRNA decreases the motivational properties of cocaine by amplifying CREB signaling, a negative regulator of the cocaine reward circuitry, and thus protects against addiction⁷⁶. At the cellular level, miRNAs such as *miR-29a/b* influence drug addiction at least partially through regulation of structural plasticity at the synapses⁷⁷.

Altogether, such studies demonstrate how desirable and pathological neuronal activity may be modulated by adult-specific functions of miRNAs. Moreover, they suggest that manipulation of miRNAs may have benefit for neurological and behavioral disorders.

Cardiovascular biology

The cardiovascular system is highly plastic and responsive to injury, stress and disease. The importance of miRNAs in this system has been demonstrated by conditional deletion of *Dicer* in the adult mouse myocardium, using tamoxifen-inducible Cre, which led to spontaneous cardiac hypertrophy, interstitial fibrosis, and reactivation of fetal gene expression⁷⁸. Ablation of miRNA biogenesis in postnatal vascular smooth muscle cells caused dramatic reduction in blood pressure due to impaired contractile function and defective vascular remodeling⁷⁹. Numerous miRNAs are involved in cardiac or vascular remodeling following stress or pathology⁸⁰. Of note are miRNAs located in the introns of different myosin heavy chain (MHC) genes (known as “myomiRs”, which include *miR-208a/b* and *miR-499*). They coordinate switching between cardiac muscle types, and their manipulation is being explored as a therapy for pathological cardiac remodeling^{81–83}.

Cardiac remodeling following pathological stresses often involves cardiac hypertrophy and fibrosis. Dozens of miRNAs are up- or down-regulated during this process, and manipulations of specific miRNAs can modulate pathological development⁸⁰. For example, the miR-34 family miRNAs are upregulated in mouse models of myocardial infarction and cardiac pressure overload via transverse aortic constriction. Subcutaneous delivery of an 8-mer locked nucleic acid (LNA)-anti-miR-34 efficiently silenced miR-34 family members and resulted in attenuated remodeling and improved heart function⁸⁴. Signalling involving calcineurin and nuclear factor of activated T cells (NFAT) controls hypertrophic growth of cardiomyocytes, and miR-23a, miR-199b and the miR-212/132 cluster members are all implicated in this signaling pathway and act as pro-hypertrophic factors. Inhibition of these miRNAs using antagomirs attenuated cardiac hypertrophy and improved heart function^{85–87}. Notably, antagomirs against miR-199b could reverse pre-existing hypertrophic phenotypes, making miR-199b a potential therapeutic target⁸⁵.

In contrast to the pathological roles of miRNAs discussed above, miR-214 is cardioprotective against ischemic injury, during which cardiomyocyte death is caused by excessive calcium influx during blood reperfusion. Despite normal baseline cardiac structure and function, *mir-214* knockout mice are more susceptible to ischemic injury and display severely impaired heart function with increased apoptosis and fibrosis after reperfusion. miR-214 antagonizes cardiomyocyte death through modulation of Ca²⁺ responses by repression of the sodium/calcium exchanger NCX1 and several other downstream effectors of Ca²⁺ signaling that mediate cell death⁸⁸.

Adult metabolism

A major challenge for adult organisms is to maintain homeostasis despite continuously dynamic metabolic states. Temporally-specific, global excision of *Dicer* in adult mice induced substantial lipid defects²⁰, and a host of individual miRNAs regulate metabolism of lipids, insulin and glucose⁸⁹.

Lipid metabolism

The most abundant miRNA in liver, miR-122, plays important roles in cholesterol and fat metabolism (see Box 3). Human *mir-33* genes are embedded within introns of sterol regulatory element binding factor (SREBF) genes, which encode key transcription factors that regulate genes involved in biosynthesis and uptake of cholesterol. Although research on SREBF genes has long focused on their central roles in regulating transcription, miR-33 was recently shown to regulate high density lipoprotein (HDL) levels by directly repressing adenosine triphosphate-binding cassette transporter A1 (*ABCA1*)^{90–93}. Treatment with miR-33 antagomirs in a mouse model of atherosclerosis caused reverse cholesterol transport and regression of the syndrome, which supports the potential therapeutic value of this miRNA⁹⁴. Similarly, inhibition of miR-33 in African green monkeys also increased plasma HDL⁹⁵. miR-33 also regulates fatty acid metabolism and insulin signaling⁹⁶.

Box 3**miR-122: Diverse links to metabolism, cancer and hepatitis**

miR-122 is specifically expressed and extremely abundant in vertebrate liver. It was targeted in the first antagomir study, which showed that upregulated genes were enriched for miR-122 sites¹⁷². Amongst downregulated genes (presumably reflecting indirect consequences) were many cholesterol biosynthesis genes, and plasma cholesterol was likewise reduced. Follow-up studies in mice and primates showed that anti-miR-122 reduced cholesterol and improved liver steatosis in diet-induced obesity models^{189, 190}. Recently, germline and liver-specific deletions of *mir-122* reproduced the reduction in serum cholesterol^{136, 137}. Interestingly, these mutants also exhibited swollen hepatocytes with abnormal lipid retention (steatosis) due to increased triglycerides, phenotypes not obtained with transient antagomir knockdowns.

Further study showed that liver stress in *mir-122* knockouts recruits immune cells that secrete proinflammatory cytokines, eventually resulting in hepatocellular carcinoma (HCC)^{136, 137}. This was rescued by injection of *mir-122* into the mutant mice at 3 months, indicating that miR-122 is an active tumor suppressor in the adult¹³⁶. Moreover, in an aggressive, non-inflammatory HCC model driven by MYC, AAV-mediated delivery of *mir-122* suppressed liver tumors¹³⁷. Thus, miR-122 suppresses cancer independently of its endogenous role in preventing hepatic inflammation. The arc of these miR-122 cancer studies is informative, since they emerged from genetic analysis of the knockout, as opposed to starting with the typical concept that this miRNA might target particular oncogenes.

The liver is the preferred site of infection by Hepatitis C virus (HCV). Although miRNAs predominantly mediate repression, miR-122 unexpectedly binds two sites in the 5' UTR of HCV to promote HCV transcript stability and translation¹⁹¹. This knowledge raises the notion of miR-122 inhibition as an HCV therapy. Extensive efforts yielded a lead inhibitor ("miravirsin") that is well-tolerated and effective at reducing HCV titers in monkeys, with no viral rebound. Miravirsin has now entered human clinical trials; it is the first miRNA-targeted drug to do so¹⁹².

Glucose homeostasis

One of the first miRNAs studied in metabolism was the pancreatic-specific miR-375, which regulates insulin secretion and glucose homeostasis^{97, 98}. Insulin resistance with disturbed glucose metabolism is a hallmark in the development of type II diabetes and obesity, and several recent studies uncovered contributions of miRNAs to these conditions. miR-143, miR-103/107 and miR-802 are all upregulated in the livers of obese mouse models as well as human patients, and hepatic overexpression of these miRNAs impaired glucose homeostasis due to attenuated insulin signaling^{99–101}. Conversely, antagomir knockdown or genetic deletions of these miRNAs improved glucose tolerance and protected mice from obesity-induced insulin resistance. More specifically, inhibition of miR-143¹⁰¹ and miR-802⁹⁹ increases insulin action in the liver while silencing of miR-103/107 predominantly enhances insulin-stimulated glucose uptake by adipocytes¹⁰⁰. The cancer-

related LIN28-Let-7 pathway also regulates glucose metabolism. Although the endogenous levels of Let-7 miRNAs are not changed in diet-induced obesity, constitutive or Dox-induced overexpression of Let-7 leads to growth retardation and glucose intolerance^{102, 103}. This is mediated in part by targeting of multiple components of insulin-PI3K-mTOR signaling in muscle cells, which are capable of glucose uptake¹⁰³. Importantly, antagomirs that block multiple let-7 members could counteract insulin resistance and lower blood glucose levels in already-established obesity state¹⁰². Finally, pharmacological inhibition of the cardiac-specific miR-208a protects mice from high fat diet-induced obesity and improves glucose tolerance¹⁰⁴. Studies such as these demonstrate the potential for manipulating miRNAs to treat metabolic syndromes.

Aging

Since metabolic pathways influence aging and are targeted by miRNAs, it is perhaps inevitable that miRNAs have been linked to lifespan and processes that change with age¹⁰⁵. Indeed, the first miRNA-target pair identified in *C. elegans*, *lin-4:lin-14*, proved relevant for longevity; overexpression of *lin-4* or depletion of *lin-14* both extend lifespan¹⁰⁶. Importantly, adult-specific knockdown of *lin-14* suppressed the short lifespan of *lin-4* mutants, with a concomitant impact on insulin signaling and accumulation of reactive oxygen species. Studies of temperature-sensitive *pasha* mutants (Box 1) revealed a phenocritical period during early adult life, during which transient loss of miRNA biogenesis results in short lifespan¹⁰⁷. Although insulin signaling is one of the best-studied aging pathways, analysis of these animals suggested an insulin-independent means by which miRNAs regulate lifespan¹⁰⁷. Interestingly, lifespan was restored by re-expressing Pasha in neurons, implying non-autonomous longevity mechanisms that perhaps involve neuroendocrine signaling. Indeed, miR-71 was recently demonstrated to act in neurons to promote organismal lifespan¹⁰⁸.

The expression of many miRNAs changes with age, and several of these miRNAs can modulate lifespan. For instance, overexpression of miR-71 and miR-246, or deletion of *mir-239* and *mir-34*, all extended *C. elegans* lifespan^{108–110}. Remarkably, the transcriptional activity of miRNAs such as *mir-71*, as inferred using promoter-GFP transgenes in live animals, was predictive of remaining lifespan¹¹¹. In mammals, miR-29 family miRNAs are substantially upregulated in multiple tissues with increasing age, as well as in a mouse model of progeria syndrome. Induction of miR-29 is associated with DNA damage responses and depends on the activation of the tumor suppressor p53¹¹². In the nervous system, Let-7b increases with age and contributes to the decline of the self-renewal capacity of neural stem cells, through targeting of the chromatin factor HMGA2¹¹³. Reciprocally, some miRNAs specifically decrease with age. For example, the immune system progressively loses its vaccination response, and this was recently connected to decline of miR-181a in naive CD4+ T cells across a 20–80 year spectrum in humans¹¹⁴. miR-181a targets *DUSP6*, which encodes an ERK phosphatase whose level increases in elderly naive T cells. Transfection of miR-181a or *DUSP6* siRNA restored the sensitivity of ERK responses in naive CD4+ T cells from elderly individuals¹¹⁴, suggesting that this regulatory interaction may potentially be exploited to improve vaccination in elderly people.

Interestingly, several recent studies of let-7 support evolutionary conserved roles for this heterochronic miRNA in animal aging. In the *D. melanogaster* testis stem cell niche, increasing age is accompanied by reduction of Unpaired, a self-renewal factor secreted by hub cells that maintains neighboring stem cells. The IGF-II mRNA binding protein (Imp) antagonizes siRNA-mediated degradation of *Unpaired* mRNA in young animals, while increased expression of let-7 across aging targets *Imp* 3'UTR and indirectly leads to Upd downregulation¹¹⁵. In *C. elegans*, let-7 restricts the regenerative capability of anterior ventral microtubule (AVM) axon in old animals through inhibition of *lin-41*¹¹⁶. Also, the let-7 family members miR-84 and miR-241 mediate lifespan-extension upon removal of the nematode gonad¹¹⁷.

Changes of miRNA expression could either serve as an adaptive response in aged animals or drive the progression of aging. For example, *D. melanogaster* miR-34 increases during aging and deletion of *mir-34* accelerates brain degeneration and shortens lifespan. Elevated expression of miR-34 improves median lifespan and mitigates neurodegeneration induced by polyQ proteins¹¹⁸. Mammalian miR-34 exhibits similar expression profiles to the fly homologue in the aging heart but, in contrast to the protective roles of *D. melanogaster* miR-34, a high level of miR-34a contributes to the age-related decline of cardiac functions. Genetic deletion or antagomir silencing of *mir-34a* significantly improved the performance of aged heart, suggesting that mouse miR-34a could be a driving factor during heart aging¹¹⁹. Profiling of brains from individuals with Parkinson's disease revealed decreased miR-34b/c, which may impact mitochondrial function¹²⁰. In contrast, patients with Alzheimer's disease and mouse models of this disease exhibit increased miR-34 in hippocampus, which has been linked to learning impairment¹²¹. These studies suggest context-dependent functions of miR-34 family miRNAs during aging and neurodegeneration. Altogether, miRNAs regulate diverse aspects of aging, at the level of the organism as well as in individual organs.

Cancer-relevant activities of miRNAs

miRNA function and dysfunction in cancer is a burgeoning area of research that has been extensively reviewed¹²². Here we highlight selected cases with evidence for adult-specific activities that directly impact cancer.

Roles for miRNAs as tumor suppressors

Several miRNAs have been suggested as tumor suppressors on the basis of their downregulation in tumors, their ability to repress oncogenes, and/or evidence that loss-of-function predisposes to cancer. Prototypical examples include the let-7 and miR-34 families. let-7 miRNAs target oncogenes such as *RAS*¹²³ and *HMGA2*¹²⁴, whereas miR-34 family members are transcriptionally activated by p53 and mediate cell cycle arrest and apoptosis^{125–127}. Both miRNA families are downregulated in several types of tumors, and their reintroduction can reduce tumor growth^{128–131}.

The existence of a dozen mammalian let-7 loci complicates the generation of genetic mouse models, but triple *mir-34a/b/c* knockouts exist^{132, 133}. As mentioned in Box 2, these mice demonstrate normal p53 responses and seemingly lack tumor susceptibility. Nevertheless,

other miRNA knockouts fulfill traditional genetic expectations for tumor suppressors. For example, *mir-15a/16-1* reside in a fragile genomic region that is frequently deleted in human chronic lymphocytic leukemia (CLL)¹³⁴, and *mir-15a/16-1* knockout mice exhibit lymphoproliferative disorders resembling CLL¹³⁵. In addition, deletion of mouse *mir-122* was recently described to drive hepatocellular carcinoma^{136, 137} (Box 2).

Especially compelling are miRNAs with selective effects on cancer cells. miR-26 is highly-expressed in liver, but downregulated in liver tumors caused by MYC overexpression¹³⁸. Forcing miR-26 expression did not affect levels of MYC, but induced G1 arrest in liver cancer cells, in part due to direct repression of Cyclins D2 and E2. Notably, in mice bearing MYC-induced hepatocyte carcinoma, delivery of *mir-26* expressed from an adeno-associated virus (AAV) vector strongly reduced cancer cell proliferation and induced tumor-specific apoptosis (Figure 4A). Importantly, this treatment did not have measurable toxicity in normal hepatocytes, which endogenously express high levels of miR-26. Therefore, miR-26 selectively arrests and kills cancer cells in established MYC-driven tumors and, moreover, functions downstream of MYC¹³⁸. Notably, miR-26a is tumorigenic in glioma and T-cell lymphoblastic leukemia, suggesting that it has tissue- or cell type-specific action during tumor development^{139, 140}.

In addition to restricting cell division and tumor growth or survival, tumor suppressor miRNAs can suppress metastasis. A key aspect of this process is the epithelial-to-mesenchymal transition (EMT), which allows cells to migrate to distant locations. Studies of the miR-200 family showed that they inhibit *ZEB1* and *ZEB2*, which are transcriptional repressors of E-cadherin, whose downregulation directs EMT^{141, 142}. Also, miR-31¹⁴³ and miR-10b¹⁴⁴ mediate opposite effects in the context of breast cancer metastasis. Acute induction of Dox-regulated *mir-31* caused regression of lung metastases established from orthotopic implantation of breast cancer cells in mice¹⁴⁵. Conversely, miR-10b antagonists suppressed lung metastases, as did retrovirally-introduced miR-10b sponges¹⁴⁴. This work on miR-10b represents a notable case where miRNA silencing by independent methods yielded *in vivo* phenocopies (see also Box 2). These studies establish principles for how miRNA activity may be manipulated to control cancer metastasis, and are being extended to other miRNAs^{146–148}.

Oncomirs

The *mir-17-92* cluster was the first characterized oncogenic miRNA (“oncomir”) locus. It is frequently amplified in human B-cell lymphomas¹⁴⁹ and cooperates with MYC to accelerate lymphoma¹⁵⁰. Recently, miR-19 was identified as the major oncogenic component of the cluster^{151–153}, simplifying hopes for miRNA silencing as a cancer therapy. However, for this to work, tumours must continuously require an oncomir. Such evidence now exists for several miRNAs.

miR-21 is upregulated in many types of human tumors¹⁵⁴ and exhibits oncogenic activity^{155, 156}. To evaluate ongoing dependency of tumors on miR-21, transgenic mice enabling conditional, tissue-specific activation of miR-21 were engineered¹⁵⁷. Following its induction at birth, ectopic miR-21 induced severe hematopoietic phenotypes, including enlarged spleen, thymus and bone marrow, concomitant with anemia and lymphoma.

Strikingly, these tumors underwent rapid apoptosis and regression following withdrawal of miR-21 (Figure 4B). This demonstration of oncomir “addiction” raises the possibility of cancer treatment by acute miRNA inhibition.

This scenario was further tested using conditional expression of miR-155, a miRNA normally involved in lymphocyte differentiation^{158, 159}. Mice in which *mir-155* is driven by the immunoglobulin heavy chain enhancer (*E μ -mir-155*) exhibit preleukemic pre-B cell proliferation that graduates to high-grade lymphoma¹⁶⁰. Recently, application of the conditional tissue-specific expression approach showed that miR-155-induced lymphoma regressed after withdrawal of miRNA expression¹⁶¹, demonstrating a second example of oncomir addiction. These models will be valuable for optimizing antagomir deliveries for cancer treatment.

Conclusions and Perspectives

This tour of adult miRNA functions highlights their influence on diverse populations of adult stem cells and differentiated cells. There are clearly commonalities between “developmental” and “adult” functions of miRNAs. For example, in both embryonic and adult stem cell lineages, miRNAs maintain homeostatic self-renewal and appropriate differentiation dynamics. Conversely, adult-specific biology provides unique demands for post-transcriptional regulation. These include long-lived cells, cells with unusual cellular architecture, and maintenance of tissue-specific and organismal homeostasis in ever-changing environments that impose physiological and behavioral stresses. We note that much work has focused on roles of miRNAs that are intrinsic to diverse types of adult stem cells, but less attention has been paid to miRNA function in niche cells whose microenvironment maintains stem cells. We expect interest in this topic to grow in the future.

In spite of tremendous multidisciplinary progress in the miRNA field, we remain hard-pressed to predict the phenotypic consequences of manipulating miRNAs *in vivo*. Although miRNAs frequently have hundreds of conserved targets, most miRNA knockouts lack overt effects on viability, fertility, visible morphology and behavior^{15, 18, 162}. Nevertheless, certain miRNA knockouts do exhibit dramatic phenotypes, as does loss of miRNA-regulation from certain target genes. Therefore, much remains to be understood about how miRNAs are incorporated into biological pathways. The growing collections of loss- and gain-of-function miRNA reagents should spur these efforts. Eventually, studies that proclaim substantial regulation of particular miRNA target genes will need to graduate towards specific manipulations of their target sites *in vivo*. “Target protectors” that occlude miRNA binding are informative and expedient¹⁶³, but target site knockouts are a worthy goal to strive for.

Understanding of continuous miRNA functions in adults may help pave the way for miRNA-based therapies, but several cautions and issues emerge. First, the substantial discrepancies between knockouts and inhibitor-based methods (Box 2) need to be resolved if miRNA-directed drugs are to become reality. The literature also shows that manipulations that are beneficial in one setting may be detrimental in another. For example, antagomirs to

let-7 and miR-122 have potential therapeutic applications for metabolic disorders and/or HCV (Box 3), but this needs to be balanced against the tumor suppressor functions of these miRNAs. The fact that many miRNAs have greater effects in sensitized conditions¹⁶⁴ highlights the need for genetic and environmental interaction studies. Indeed, we discussed many cases of unexpectedly desirable consequences of manipulating miRNAs in the context of disease, as highlighted by the fact that some miRNA loss-of-function conditions appear normal yet confer protective or beneficial in pathological conditions. On the other hand, systematic studies of inducible *D. melanogaster* miRNA transgenes uncovered scores of surprisingly specific, dominant phenotypes^{165, 166}. These surveys suggest that miRNA gain-of-function may generate disease much more frequently than miRNA loss-of-function, and should encourage the generation of similar resources in mice.

Perhaps the ultimate challenge continues to be achieving effective delivery of miRNA mimics or antagomirs. The liver is favorably targeted by circulating drugs and external tissues and the digestive tract are also easily accessed. However, small RNA drugs must cross cell membranes and eventually incorporate into mature Argonaute complexes to mediate function. Having knowledge of clear phenotypic and quantitative readouts of efficacy will be invaluable to judge improvement in potency and efficacy of small RNA drugs.

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GLOSSARY

Cre	A site-specific recombinase that recognizes a specific sequence termed loxP. The DNA located between two loxP sites is excised following Cre-mediated recombination
Drosha	An RNase III enzyme that cleaves primary miRNA transcripts (pri-miRNAs) into hairpin structure called pre-miRNAs, which become substrates for Dicer processing
Pasha	A double-stranded RNA binding protein that complexes with Drosha to cleave primary miRNA transcripts into pre-miRNA hairpins. It is called DGCR8 (DiGeorge syndrome chromosomal region 8) in vertebrates
Dicer	An RNase III enzyme that processes pre-miRNA hairpins or long double-stranded RNAs (dsRNAs) into dsRNA duplexes of ~21–22 base pairs
Argonaute	A family of effector proteins that bind small RNAs (such as miRNAs) and mediate their regulatory effect on target transcripts

Leptotene	The first stage of meiotic prophase. Chromosomes begin to condense
Zygotene	The second stage of meiotic prophase. Homologous chromosomes pair
meiotic sex chromosome inactivation (MSCI)	The transcriptional silencing of the X and Y chromosomes during the meiotic phase of spermatogenesis
transit-amplifying cells	A type of rapidly-dividing progenitor cell capable of dividing a finite number of times before differentiation
long term potentiation	Activity-dependent, long-lasting enhancement of synaptic transmission between neurons
contextual fear memory	A form of fear memory established through association of aversive stimuli (e.g. electric shock) with a particular neutral context
GW182 proteins	Glycine–tryptophan repeat-containing proteins that interact with the microRNA-induced silencing complex (miRISC) to recruit proteins that mediate degradation or translational repression of target mRNAs
locked nucleic acid Locked nucleic acids (LNAs)	A class of RNA analogues in which the 2' oxygen and the 4' carbon positions in the ribose ring are connected or 'locked' to create increased thermal stability relative to DNA or RNA when they are complexed with complementary DNA or RNA
Heterochronic gene	A gene that determines an organism's stage-specific temporal state during its development
hub cells	10~15 somatic cells residing at the apical tip of the <i>Drosophila</i> testis, which contact and maintain the neighboring germline stem cells
oncomir addiction	Continuous dependence of tumor cells on the oncogenic miRNA (oncomir) for the maintenance of the malignant phenotype
miRNA seed sequences	The nucleotides ~2~8 at the 5' end of a miRNA. miRNAs function predominantly through base pairing between the seed region and target mRNA 3' UTR

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Biographies

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Eric C. Lai is a Member of the Developmental Biology program at Sloan-Kettering Institute. His early work on post-transcriptional control of Notch signaling during *Drosophila* neurogenesis established regulatory principles of miRNA targeting. His laboratory continues to study these topics and has extended these efforts to mammalian neural stem cell systems. In addition, his group is interested in small RNA annotation and elucidation of non-canonical miRNA biogenesis pathways.

KEY POINTS

- miRNAs are actively involved in diverse aspects of adult physiology, including in stem cells, in the nervous system, cardiovascular system, and during metabolism and aging.
- In the context of cancer development, miRNAs could serve as either tumor suppressors or oncogenes in a tissue dependent manner.
- Continuous functions of miRNAs during adulthood and availability of methodologies for miRNA manipulation make miRNAs promising as therapeutic targets and agents.
- We evaluate the strengths and weaknesses of current methods to study miRNA functions specifically in the adults.

Figure 1. Major strategies for analyzing miRNA loss of function in vivo

These techniques are generally used to study gene functions during development, but they can also be used to manipulate gene activity specifically during adult stages. (A) In *Drosophila*, mitotic recombination mediated by FLP recombinase and FRT target sites generates homozygous mutant cells. Recombination can be temporally controlled in the adult using heat-shock inducible FLP. (B) In mice, excision of loxP cassettes by Cre recombinase generates a deletion allele. Temporal control is afforded using tamoxifen-inducible Cre, or by using Cre lines that are active only in adult stages or that are sensitive to environmental manipulation. In both fly and mouse systems, one can analyze individual miRNA loci or miRNA biogenesis factors. (C) In *C. elegans*, a temperature sensitive allele of the core miRNA biogenesis factor *Pasha* permits temporal, global, and reversible inhibition of miRNA synthesis in the intact animal. (D) Several types of miRNA inhibitors have been developed. These include “sponges” that typically consist of a reporter linked to

multiple target sites for a given miRNA, “tough decoys” that contain two miRNA binding sites within a structured non-coding RNA, and “antagomirs” that are antisense miRNA sequences built on a modified backbone that incorporates locked nucleic acids (LNAs) for stability and can include cholesterol conjugation to enhance cell uptake. These inhibitors or inhibitor constructs can be injected directly into adults or, in the case of sponges, expressed using temporal control.

Figure 2. miRNA function in adult stem cell lineages

(A) Many adult tissues are replenished throughout lifetime by stem cells located in specific niches. This schematic depicts the major sites of mammalian adult stem cells. Highlighted are stem cell niches that have been studied extensively with respect to their requirement for miRNAs, and most of these are discussed in the text. Thus far, little study has been made of intestinal stem cells in this context. (B) Paradigms for miRNA function in stem cell lineages. miR-184 controls the balance between proliferation and differentiation of adult neural stem cells, in which high levels of miR-184 promotes proliferation and inhibits differentiation through regulation of *Numbl*. In muscle satellite cells, expression of miR-1 and miR-206 increase and represses the transcription factor Pax7 as the cells enter the differentiation program. In the hematopoietic system, miR-223 is a negative regulator of granulocyte progenitor proliferation and differentiation. Notably, there is a steady increase of miR-223 level as the hematopoietic stem/progenitor cells adopt the granulocytic cell fate, and differentiated neutrophilia lacking miR-223 exhibit hypermature and hyperactive phenotypes, indicating the requirement of miR-223 in multiple stages during granulocyte differentiation.

Figure 3. Multiple functions of an individual miRNA in adult neural differentiation and function

The nervous system-specific miRNA miR-124 is absent from neural stem cells (NSCs) but is expressed in their differentiating progeny, starting in transit-amplifying progenitor (TAP) cells and increasing in neuroblasts (NBs) and mature neurons. miR-124 promotes neural differentiation, in part by directly repressing the NSC determinant SRY-box containing 9 (*Sox9*). Inhibition of miR-124 impairs adult neurogenesis by impairing cell fate transitions, as indicated. Ectopic miR-124 promotes premature neuronal differentiation yielding a transient increase in neurogenesis. This effect fades as the NSC pool is depleted via the loss of self-renewing divisions. (B) miR-124 is maintained in mature neurons and affects neural function and behavior. Double knockouts of the genes encoding the cAMP binding proteins EPAC1 (also known as Rap guanine nucleotide exchange factor 3, RRGF3) and EPAC2 impair long term potentiation (LTP) and cause defective learning and social interactions⁶⁹. Remarkably, miR-124 is not only transcriptionally upregulated in *EPAC* mutants, but substantially mediates their phenotype. Delivery of miR-124 antagomirs did not affect synaptic transmission, but restored LTP and normal behavior in *EPAC* mutants. Reciprocally, adenoviral-mediated overexpression of miR-124 in adult hippocampus and prefrontal cortex, respectively, induced learning and social behavior phenotypes characteristic of *EPAC* mutants⁶⁹. Finally, adult knockdown of the miR-124 target early growth response 1 (*Egr1*, also known as *Zif268*) phenocopied behavioral defects of miR-124 overexpression, implicating it as a key target in this pathway.

Figure 4. Active and continuous roles for miRNAs in cancer

In concept, miRNAs could act as tumor suppressors by targeting oncogenes, or as oncogenes by repressing tumor suppressor genes. In practice, powerful evidence for the involvement of miRNAs in cancer can be obtained using genetics, irrespective of whether the relevant target genes are known. Particularly compelling are examples of miRNAs whose active involvement in adult-stage cancer is demonstrable. (A) Example of a tumor suppressor miRNA. Kota and colleagues used a model of hepatocellular carcinoma (HCC) driven by conditional liver-specific expression of *Myc*¹³⁸. miR-26 is normally high in the liver, but is downregulated upon MYC induction. Following the development of liver tumors, injection of adeno-associated virus (AAV) vector engineered to express miR-26 is able to cause HCC regression, even though MYC expression is maintained. (B) Example of a tumor addition to an oncogenic miRNA. Slack and colleagues engineered mice that carry inducible *mir-21* under tissue-specific and temporal control¹⁵⁷. Induction of miR-21 in the hematopoietic system caused lymphoma, amongst other phenotypes, demonstrating that this miRNA is sufficient to initiate cancer. Genetic shut-off of miR-21 expression causes regression and apoptosis of the tumors, demonstrating that overexpression of this miRNA is also actively required for tumor maintenance.