Emerging role of microRNAs in disease pathogenesis and strategies for therapeutic modulation

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Micro (mi)RNAs are small, regulatory RNA molecules that are integral components of the genetic program in the majority of cells. They are thought to regulate up to one third of all human genes and have been linked to critical processes in disease pathogenesis. The diverse role of miRNAs in disease pathogenesis suggests that the modulation of miRNA function by utilizing techniques such as the use of antagonism, locked nucleic acids, or miRNA sponges may produce novel therapeutic approaches. In this review, the current understanding of post-transcriptional gene regulation by miRNAs is discussed and insights into the function of miRNAs in tumorigenesis, immune responses, muscle function, organogenesis, and cell-lineage decisions are reviewed.

Keywords: Antagonism, miRNA, post-transcriptional gene regulation

Introduction

Post-transcriptional inhibition of translation by miRNAs

Micro (mi)RNAs are small (approximately 22 nucleotides) regulatory RNA molecules. They are integral components of the genetic program in the majority of cells and are also expressed by some viruses [1-6]. Their genes are localized within the introns of protein-coding genes or non-coding regions of the genome [1,2,7-9]. Since the discovery of the first miRNA, Lin-4, over a decade ago [10], thousands of small 'non-messenger' RNA molecules have been identified and are tabulated in miRNA registries [11-14]. However, only a limited number of miRNAs, many of which are ubiquitously expressed, account for differences in miRNA profiles between diverse cell types and tissues. Landgraf et al cloned and sequenced approximately 330,000 RNA sequences, which were derived from 256 small RNA libraries prepared from human and rodent cell lines and organ tissues, and identified 416, 386 and 325 miRNA precursor genes present in the human, mouse, and rat genomes, respectively [15**]. Most of these miRNAs were highly conserved between species and less than a third were identified at high expression levels or expressed with a high degree of tissue specificity [15**]. Therefore, the number of miRNAs with functional relevance in human and animal cells may be far less than previously estimated.

A complex set of proteins is required for the processing of long primary miRNA molecules into stem-loop precursors, their active transport into the cytoplasm and cleavage into miRNA duplexes of approximately 22 nucleotides in length [1,4,6,16-24]. In the cytoplasm the functional strand of the mature miRNA duplex may dissociate from its complementary non-functional strand and locate within the RNA-induced silencing complex (RISC), composed of Dicer RNAse III (Dicer), its partner transactivation response RNA-binding protein, and Argonaute protein, while the passenger strand is degraded (Figure 1) [25]. In contrast with small interfering (si)RNAs, the functional miRNA strand is usually not fully complementary to the sequence of its target miRNAs, and recognition of its cognate targets is guided by a seven-nucleotide site, termed the seed sequence, in the 5' end of the miRNA [26,27]. The 3' region of the miRNA may be partially zipped up with the free 5' regions of the target miRNA [1]. However, seed matches alone guided by the base-pairing rules of Watson and Crick are not always sufficient for binding to miRNA. Target factors that boost miRNA efficacy include AU-rich nucleotide composition near the miRNA binding site, positioning of the binding site within the 3' untranslated region (UTR) but away from the center of long UTRs, proximity to binding sites of other miRNAs to facilitate cooperative action, and the amount of mRNA and miRNA complexes [28-30]. Despite the complexity of target recognition, computational evidence suggests
Figure 1. Post-transcriptional inhibition of translation by miRNAs.

The functional strand of the mature micro (mi)RNA duplex locates within the RNA-induced silencing complex (RISC), which is composed of Dicer RNase III (Dicer), transactivation response RNA-binding protein (TRBP) and Argonaute 2. Recognition of its cognate mRNA targets is guided by a seven-nucleotide site in the 5' end of the miRNA, termed the seed sequence. Binding produces translational repression. 18s 18s ribosome; 28s 28s ribosome.


that as many as 200 genes may be regulated by a single miRNA [31]. Notably, several miRNAs may be required to bind to one mRNA target for combinatorial control of protein translation [1]. Therefore, miRNAs are proposed to regulate up to one-third of all human genes at the post-transcriptional level [32]. While miRNA targets are still ill defined, computer-based tools are readily available to predict the target genes of a respective miRNA based on the complementarity to its seed sequence [31-40]. Reliability in the identification of miRNA targeted by unique miRNAs was improved by additional evaluations of energy states of sequences flanking the miRNA and the presence of stabilizing elements within the miRNA [41,42]. For example, biochemical identification approaches, where Argonaute proteins associated with specific miRNA were co-immunoprecipitated with target mRNA, showed a high concordance with computer algorithm-based target predictions [43].

Initially it was thought that miRNAs either degrade the newly synthesized protein as it emerged from the ribosome or 'freeze' the ribosome during translation [44]. However, when bound to its target, the miRNA-RISC complex was shown to block the initiation of translation (specifically the 5' cap recognition process), but no destabilization of the transcript was observed [45-47]. Subsequently, miRNA binding was demonstrated to promote the movement of the mRNA from the cytosol to sites of RNA degradation, termed 'P-bodies' [46,47]. This was achieved by RISC through the removal of the 5' 7-methylguanosine cap, a characteristic of mRNA molecules and a prerequisite for their destruction in P-bodies [47,48]. While P-body components play crucial roles in mRNA decay, aggregation into P-bodies may not be required for miRNA function, but might instead be a consequence of their activity [49]. This pathway is presumed to be distinct from that of the siRNA-directed cleavage pathway, where RISC first cleaves the target mRNA into small fragments before degradation in exosomes. However, some miRNA, can expedite poly(A) tail removal to facilitate decay of the transcribed portion of miRNAs, even if they contain elements that are imperfectly complementary to the miRNA sequence [50]. This increased rate of deadenylation of target mRNA does not result from blocking of translation, and conversely poly(A) removal may not be required for translational repression by miRNA [50]. In addition, these distinct regulatory influences of miRNA, namely translational repression versus mRNA decay, are thought to be mediated by similar protein complexes that deliver them to their mRNA targets [17,22,51-54].

Role of miRNA in diverse biological processes

In vitro and in vivo studies and clinical trials have identified miRNAs as important regulators of a broad spectrum of biological processes, including tumorigenesis, aberrant immune responses, muscle dysfunction, and organogenesis. In this review, key functions of unique miRNAs thought to be relevant in disease pathogenesis are highlighted to facilitate the integration of the emerging understanding of miRNAs with novel therapeutic approaches.

miRNAs and tumorigenesis

miRNA expression profiling utilizing 98 different small RNA libraries, including sorted cell populations from healthy donors, whole bone marrow, cell lines, and tumor cells isolated from patients with hematological malignancies, demonstrated that only five miRNAs are highly specific for hematopoietic cells: miR-142, miR-144, miR-155, miR-223 and miR-150 [15-4]). However, miR-150 expression was markedly reduced in B-cell lymphomas [15++, and when
ectopically expressed this miRNA inhibited the early stages of B-cell development [55]. Therefore, deregulated hematopoiesis by miRNAs may promote malignant transformation.

Distinct miRNA signatures have also been associated with prognostic factors and disease progression in chronic lymphocytic leukemia (CLL) [56,57]. Reduced expression of miR-29 and miR-181 in patients with CLL correlated with high expression of the oncogene T-cell leukemia-lymphoma 1, which is thought to be a causal event in the development of the aggressive form of this disease [57]. The miRNA gene clusters miR-15a to miR-16-1 and miR-24-1 to miR-23b were also abnormally expressed in CLL and the target of some of these miRNAs is the anti-apoptotic BCL-2 gene, which is overexpressed in the majority of CLL patients [56]. Therefore, miRNAs function as important tumor suppressors and reduced expression may promote malignant transformation [58,59].

Disturbances of miRNA expression may also play a role in the initiation and progression of solid tumors. Many studies demonstrated that miRNA profiles are dramatically shifted in these tissues. For example, miRNA signatures in pancreas tissues were able to distinguish adenocarcinomas from normal samples [60]. In addition, expression profiling in glioblastomas revealed high levels of miR-21, miR-221 and miR-222 [61], and knockdown of miR-21 in those cells triggered activation of caspases and led to increased apoptotic cell death [62]. High expression of miR-21 may also downregulate the tumor suppressor tropomyosin 1 in breast cancer cells, thereby promoting tumorigenesis [63]. Some miRNAs (miR-26, -107 and -210) upregulated in tumor tissues are only found in a hypoxic environment, where they decrease pro-apoptotic signaling [64]. A group of miRNAs, which share sequence identity with miR-16, negatively regulates cellular growth and cell cycle progression [65]. A number of miRNA genes are located in regions of genomic instability or cancer susceptibility loci, supporting a possible role of altered miRNA expression in tumorigenesis [66,67]. Therefore, miRNA profiling might improve diagnosis and predict prognosis of malignancies and suggests that miRNA may play a key role in tumorigenesis by regulating tumor suppressors, apoptosis, and cell cycle progression.

miRNAs in immune responses

Another 'oncomir' specific for hematopoietic cells, miR-155, may be implicated in the regulation of adaptive immune function [68]. Mice deficient for miR-155 not only displayed Th2 cell activation, but also a dysfunction of B-lymphocytes and dendritic cells [69•]. This produced an impaired antibody response, lung inflammation, and airway remodeling [69•]. By analyzing the transcriptome of miR-155-deficient CD4+ T-cells, a wide spectrum of miR-155-regulated genes, including Th2 cytokines, chemokines, and transcription factors, were identified [69•]. Expression of miR-155 was induced by several cytokines as well as TLR ligands that are important pathogen recognition molecules [70]. Therefore, miR-155 is a target of a broad range of inflammatory mediators that promote humoral and cellular immune responses to microbial infections, and in its absence a marked immune dysfunction develops. A role of miRNAs in T-cell function is additionally supported by studies in mice lacking Dicer (a protein required for miRNA function) in the T-cell lineage. These mice were not only depleted of all miRNAs in their T-cell lineage, but were also prone to immune pathologies because of an impaired regulatory T-cell function, demonstrating a role for miRNAs in the regulation of immune responses [71].

Altered miRNA expression profile has also been observed in chronic inflammatory diseases. For example, miR-203 was upregulated in inflammatory skin lesions of patients with psoriasis and this was associated with reduced levels of an evolutionary conserved target of miR-203, suppressor of cytokine signaling 3, which regulates inflammatory responses and keratinocyte functions [72]. Therefore, miRNA deregulation may contribute to dysfunction of the cross-talk between resident and infiltrating immune cells [72].

LPS as well as antigen challenges are associated with rapid disturbances in miRNA levels in the mouse lung, suggesting that these changes might modify the subsequent development of inflammatory and allergic responses [73,74]. Interestingly, LPS-induced miRNA expression was not altered by anti-inflammatory glucocorticoid treatment, suggesting that some miRNA effects may not be mediated via classical steroid-sensitive signaling cascades [73]. Therefore, while knowledge is still limited, there is a clear link between aberrant miRNA expression and important pathways of the innate and adaptive immune response.

miRNAs and viruses

Viruses also encode miRNAs and appear to evolve rapidly and regulate both the viral life cycle and the interaction between viruses and their hosts [75,76]. For example, the CMV miRNA, hcmv-miR-UL112, targets a host transcript that is important in natural killer (NK)-cell activation (NK-cell activating receptor MHC-I-related chain B), and inhibition of hcmv-miR-UL112 increased killing of CMV-infected cells by NK-cells [77]. Viral miRNAs may also target their own viral transcripts. Among 18 miRNAs encoded by EBV, 1 miRNA repressed viral replication during infection by targeting viral polymerase [78]. Therefore, viruses appear to utilize miRNAs for immune evasion.

Conversely, miRNAs derived from host cells may also limit viral replication. miR-32 targets an mRNA encoded by the primate foamy virus type 1 (PFV-1) and thereby restricts its replication [79,80]. PFV-1 produces a protein (Tas), which may interfere with the miRNA pathway of the host [79•]. In addition, host miRNA may be utilized for viral replication [81•]. Thus, miRNAs are an integral part of the antiviral response, and could serve as diagnostic markers and novel antiviral targets.

Muscle function and miRNAs

Hypertrophic growth and fibrosis of the cardiac muscle were accompanied by disturbances in the levels of the cardiac-specific miR-208 that regulate the expression of the primary
contractile proteins of the heart, the α- and β-myosin heavy chain [82••]. The muscle-specific miR-1 and miR-133 may also promote cardiac hypertrophy [83•,84], and miR-1 was upregulated in patients with coronary artery disease and associated with the development of arrhythmias [85]. For example, the systemic neutralization of miR-133 by an 'antagomir' led to a cardiac hypertrophy [83•]. Targets of miR-133 include RhoA, a GDP-GTP exchange protein regulating cardiac hypertrophy; Cdc42, a signal transduction kinase implicated in hypertrophy; and Nef-negative elongation factor A/Wolf-Hirschhorn syndrome candidate 2 protein, a nuclear factor involved in cardiogenesis. Reduced miR-133 expression favored apoptosis by activation of caspase-9 [86]. Therefore, miRNAs are key regulators of cardiac muscle cell function and survival.

miRNAs in organogenesis and cell-lineage decision

Many miRNAs are upregulated during development in a tissue-specific manner, suggesting a broad involvement or role for miRNAs in organogenesis, cell-lineage decisions, and the capacity for self-renewal [87-90]. This is highlighted by a study where depletion of Dicer in oocytes blocked their progression through the first cell division [91]. Thus, miRNAs may control translation of maternally derived genes in the earliest developmental stages of the embryo.

Hematopoietic cells demonstrate different miRNA expression patterns when compared with non-hematopoietic cells. Differentiated effector cells of the hematopoietic lineage (eg, Th1 and Th2 cells and mast cells) and precursors at comparable stages of differentiation (eg, thymocytes and pro-B-cells) show similarities in their miRNA profile [15••,92]. Moreover, miRNA levels may also determine the further differentiation of the hematopoietic stem cell into effector cells. For example, early B-cell development may be regulated by miR-150 and monocytopenia by several miRNAs, including miR-17, miR-20a and miR-106a [55,93].

miRNAs are essential for normal muscle cell development in mice. Blocking the biogenesis of miRNAs by inactivation of Dicer in skeletal muscle resulted in hypoplasia and abnormal morphology of myofibers [94]. Dicer activity was also required in the pancreas during embryogenesis [95]. Excess of miRNAs, specifically miR-1, during embryogenesis impaired the proliferation of cardiomyocytes through downregulation of the transcription factor Hand2 [41].

During lung development several miRNAs are differentially expressed; some are maternally imprinted (eg, miR-154 and miR-335) and located on human chromosome 14q32.31 (mouse chromosome 12F2) [96]. This miRNA expression profile was highly conserved between human and mouse lung, again highlighting the importance of miRNAs in organogenesis. Of note, individuals with both chromosome 14 alleles inherited from the father (paternal uniparental isodisomy chromosome 14), and therefore complete absence of the maternally imprinted miR-154 and miR-335 families, among other imprinted genes, exhibit a severe lung hypoplasia [97].

Sequence variations in miRNA genes and target sites

Sequence variations have been identified in human miRNA precursor genes or their 5' flanking region, presumably the miRNA promoter region [98]. Germ-line or somatic mutations were observed in 5 of 42 sequenced miRNAs in 11 of 25 chronic lymphocytic leukemia patients, but not in 160 healthy individuals [56•]. While sequence variations are rare in miRNA precursor genes, a higher level of variation was observed at miRNA target sites [99]. Approximately 400 SNPs were present at those target sites that are otherwise evolutionarily conserved across mammals. Most may be of functional relevance, as they could create novel target sites for miRNAs in humans [99]. Another genome-wide analysis of SNPs located in miRNA target sites showed that twelve SNPs were associated with human cancers [100].

An SNP localized in the 3' UTR of the myostatin GDF8 gene created a novel target site for miR-1 and miR-206, and was associated with muscle mass in sheep [101]. In separate studies, this gain-of-function mutation caused post-transcriptional inhibition of myostatin contributing to the muscular hypertrophy [102].

The effect of an SNP (rs5186) in one of the target sites for miR-155, located within the 3' UTR of the human angiotensin II type-1 receptor gene, was investigated using reporter silencing assays [103]. mir-155 downregulated the expression of the 1166A and not the 1166C allele of rs5186. The 1166C allele has been associated with hypertension, thereby linking mir-155 to regulation of blood pressure [103]. Taken together, SNPs located in miRNA precursor genes and their target sites can affect miRNA target expression and function and may be associated with phenotypic differences, for example, cancer, muscular hypertrophy and high blood pressure.

Inhibition of miRNA function

The use of synthetic antisense analog of miRNAs termed 'antagomirs' has shown that cells expressing miRNAs of interest can be targeted in vivo [104••]. Antagomirs are cholesterol-conjugated single-stranded RNA molecules of 21 to 23 nucleotides in length and entirely complementary to the functional miRNA strand (Figure 2). They silence miRNA expression in the liver, lung, intestine, heart, skin and bone marrow for over a week following intravenous injection, and thereby regulate the expression of genes specifically predicted to be controlled by the respective miRNA [83•,104•,105].

Locked nucleic acids (LNAs) are RNA oligonucleotides in which the ribose moiety is modified with an extra methylene bridge, connecting the 2' with the 4' carbons to produce increased metabolic stability, high affinity, improved mismatch discrimination, and low toxicity [106]. LNAs are currently utilized to inhibit miRNAs in cultured cells and as probes for miRNAs [107], but they are a particularly attractive candidate for in vivo modulation of miRNA function.

Another approach to inhibit miRNAs is the use of synthetic miRNAs that contain multiple binding sites for a specific miRNA, thereby inhibiting its association with the endogenous target [108**]. To prevent cleavage of the miRNA containing the miRNA binding site via the RNA interference pathway, mismatches were introduced at position 9 to 12, in close proximity to the seed sequence. These 'micro RNA sponges' depressed miRNA targets at least as strongly as chemically modified antisense oligonucleotides in cultured cells [108**]. The use of inducible and tissue-specific miRNA sponges driven by RNA polymerase promoters and the generation of transgenic animals expressing inducible sponges may be feasible in the future.

Numerous RNA-binding proteins, only one of which is miRISC, target the 3' UTRs of miRNAs. Intriguingly, by binding to target miRNAs the RNA-binding protein Dnd-1 blocked their interaction with miRNAs and thereby protected miRNAs from miRNA-mediated repression of initiation of translation [109]. Thus RNA-binding proteins that are not directly part of the miRNA complex, such as Dnd-1 or Hu antigen R, may be utilized to fine-tune miRNA-mediated silencing [109,110].

Overexpression of miRNAs
As discussed above, many tumors exhibit reduced miRNA levels and it may be possible to restore those levels by creating synthetic miRNAs or using gene-therapy based approaches. This could be achieved by nonviral or viral delivery of the miRNA/transgene ex vivo or in vivo. As experience with these delivery techniques accumulates, the understanding of miRNA regulation is improving and therefore maintaining normal miRNA levels in specific tissues is becoming increasingly feasible.

Conclusion
Diverse roles of miRNAs in tumorigenesis and apoptosis, immune and inflammatory responses, muscle differentiation and function, and organogenesis have been suggested. miRNA profiling can also be used as a novel diagnostic and prognostic marker of disease. The possibility of modulating miRNA levels in vivo is likely to lead to a better understanding of the fundamental mechanisms that underpin the development of a broad range of diseases and may ultimately produce novel therapeutic approaches [111,112]. Major challenges in the field of therapeutic miRNA modulation include the development of efficient and non-toxic delivery techniques, the prevention of off-target silencing effects, and the maintenance of physiological miRNA levels in gene-targeted tissues.

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References
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- * of special interest

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80. This study showed that cellular miRNAs were expressed to limit viral replication.


83. This study showed that miR-122 promotes viral replication.


85. This study demonstrated protection from cardiac fibrosis and hypertrophy in mice deficient in miR-200.


87. This study demonstrated that inhibition of miR-133 with antagonists results in cardiac hypertrophy.


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** This study utilized specific inhibitors of miRNAs, termed antagonism, in vivo and demonstrated an important role for miRNAs in metabolic processes.


** This study transgenically expressed RNAs bearing multiple miRNA target sites for miRNA neutralization.


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