

PATHOBIOLOGY IN FOCUS

Everything you wanted to know about small RNA but were afraid to ask

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MicroRNAs are a class of recently discovered small RNA molecules that regulate other genes in the human genome. Studies in human cells and model organisms have begun to reveal the mechanisms of microRNA activity, and the wide range of normal physiological functions they influence. Their alteration in pathologic states from cancer to cardiovascular disease is also increasingly clear. A review of current evidence for the role of these molecules in human health and disease will be helpful to pathologists and medical researchers as the fascinating story of these small regulators continues to unfold.

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Modern medicine has begun to gather an understanding of the molecular mechanisms that give rise to the cellular patterns long seen through the pathologist's microscope. The 'central dogma' of DNA genes copied to messenger RNA transcripts that guide the synthesis of proteins still provides the basic framework connecting genetics to medical phenomena. Yet, as is often the case in biology and medicine, the details and exceptions can be of particular interest.

The recently discovered tiny RNA molecules known as microRNAs are important regulators of the other genes in the human genome. Our understanding of these small RNA molecules is still in its infancy, but examples from human cells as well as mice and other model organisms indicate that microRNA-mediated gene regulation is likely to play key roles in human development, cellular differentiation, adaptation to the environment, oncogenesis, and host cell interactions with pathogens. For pathologists, methods for measuring microRNA levels offer a new set of tools that may be of use in improving our understanding and classification of many diseases.

DISCOVERY OF SMALL RNA

Previous decades unveiled a number of important cellular roles for RNA distinct from the information-encoding transcript of messenger RNA. Ribosomal RNA is both a structural and catalytic part of the ribosome;¹ tRNAs play an informational role in decoding the codons of the messenger

RNA while carrying amino acid residues for protein synthesis;² the small nuclear RNAs of the spliceosome recognize splice sites and catalyze alternate splicing of pre-mRNA transcripts;³ and several small nucleolar RNAs are involved in covalent modification of other RNA bases.⁴ To continue the list, the 7SL RNA is a part of the signal recognition particle that channels proteins into the endoplasmic reticulum during synthesis;⁵ the telomerase complex carries its own RNA as a template for extending the ends of chromosomes;⁶ and the very long noncoding RNA XIST is required for X-chromosome silencing and dosage compensation in human females.⁷

The discovery of very short noncoding RNAs as regulators of gene activity began almost 15 years ago when researchers studying larval development in the nematode worm *C. elegans* discovered a gene, *lin-4*, that encoded short RNA transcripts able to inhibit translation of the messenger RNA of a different gene, *lin-14*, by binding to its 3' untranslated region.⁸ In hindsight, *lin-4* was the first of the microRNA class of genes to be characterized. We now realize that there are hundreds of similar microRNA genes in the human genome. However, before the widespread importance of microRNAs came to be appreciated, another kind of small RNA molecule took center stage.

Geneticists studying pigment production in petunias in the late 1980s made the paradoxical observation that plants carrying extra copies of a pigment production transgene

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sometimes produced flowers with patchy pigment, or little pigment at all.^{9,10} The mechanism for this trans-acting 'co-suppression' silencing of the endogenous pigment production gene was not fully known, but RNA produced by the transgene array was a prime suspect as a mediator of silencing.

Shortly thereafter, researchers studying the inconsistent ability of injected RNA to silence genes in *C. elegans* tested whether sense-, anti-sense, or double-stranded RNA homologous to a gene was the most active in suppressing endogenous gene activity. Surprisingly, double-stranded RNA had the greatest silencing effect.¹¹ Further investigations by several labs revealed that short (21–22 nucleotide) double-stranded RNA cassettes (short interfering RNA, or siRNA) were generated from longer double-stranded RNA by an enzymatic system within the cell, and functioned by causing cleavage of the endogenous gene mRNA to which they were homologous.^{12–15} Although human cells have not yet been shown to generate endogenous siRNAs, the evolutionarily conserved enzymatic machinery used with microRNAs can recognize siRNAs. As a result, exogenous siRNA reagents can be used to 'knock down' human gene function for research, and potentially for therapeutic purposes.

Spurred on by these findings, a large number of other researchers have begun to flesh out the picture of small RNA biology in human cells. Identification of short RNA molecules from a variety of healthy and diseased human tissues indicates that there may be well over 500 distinct microRNAs in the human genome.^{16,17} Some of the roles of these microRNAs in normal human biology and in disease are beginning to come into focus.

Notably, entirely new classes of small RNA molecules such as the PIWI-associated RNAs (piRNAs) in the male germline indicate that even the broad outlines of this subject have not yet been fully delineated.¹⁸ Results from the latest generations of genome-tiling microarrays and high-throughput sequencing indicate that there may be a considerably richer universe of non-coding RNAs of various sizes, produced from many different regions of the genome, than had been previously supposed.^{19–21} In addition, comparisons between animals with increasingly complicated bodies have been used to suggest that the proportion of non-coding RNA to protein-coding RNA is correlated with developmental complexity.^{22–24}

MicroRNAs

Mature microRNAs are single-stranded RNA molecules that are approximately 21 or 22 nucleotides long. Most microRNAs are generated from primary transcripts (pri-miRNAs) produced by RNA polymerase II, the same RNA polymerase that transcribes protein-coding genes, but some microRNAs in repetitive regions of the genome are transcribed by RNA polymerase III.^{25–27} Some microRNA primary transcripts encode only a single mature microRNA, while other loci contain clusters of microRNAs that appear to be produced from a single primary transcript. Approximately half of all human microRNA genes are contained within the introns of

protein-coding genes, while others reside apart from known genes, or in the exons of untranslated genes.²⁸ Control of pri-miRNA synthesis is incompletely understood, but some of the same transcription factors that regulate protein-coding genes can apparently also regulate pri-miRNAs.²⁹

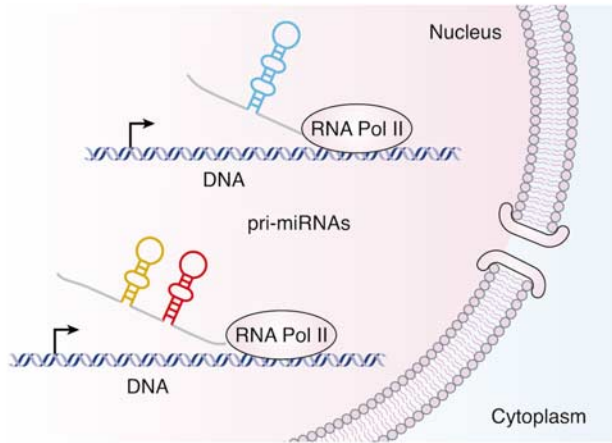
Within the primary transcript, the microRNA-encoding portions form imperfect stem-loop hairpin structures. A nuclear protein complex called the 'Microprocessor,' consisting of the Drosha type III RNase, the double-stranded RNA-binding protein DGCR8, and a number of other proteins, cleaves the hairpin-loop pre-miRNAs away from the rest of the primary transcript, permitting export of pre-miRNAs through the nuclear pore to the cytoplasm.³⁰ There, the pre-miRNAs are further processed by a protein complex containing the Dicer type III RNase that cleaves the microRNA to its mature size.^{31,32} In general, only one strand of the original precursor is kept as a mature microRNA. The mature microRNA becomes associated with a final protein complex called the miRISC (microRNA-induced silencing complex), which typically contains an Argonaute family protein, and which carries out the silencing of the genes targeted by the microRNA (Figure 1). Within many miRNA sequences, the so-called 5' 'seed' region (from base 2 to base 8 or 9) is particularly important for target site recognition, but sequence context and base pairing between regions in the 3' half of the microRNA and the target site can also contribute to the function of the microRNA.^{33–35}

It appears that at least in some cells, the processing steps required to generate mature microRNAs from pri-miRNA transcripts are actively regulated, which may give extra flexibility for controlling the amount of active microRNA in the cell.³⁶

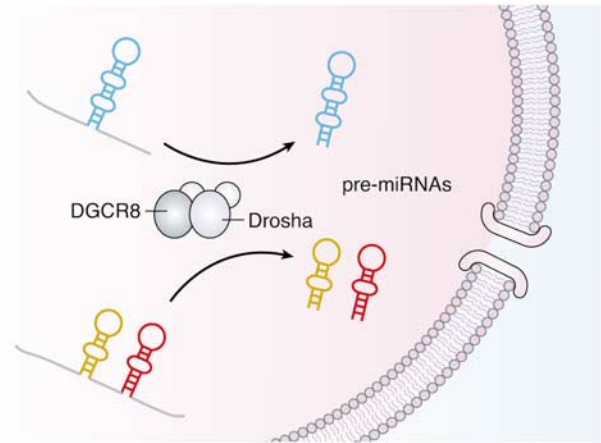
Mechanisms of MicroRNA Action

Unlike siRNAs, which require near-perfect complementarity to the messenger RNA they target for cleavage, most microRNAs form imperfectly complementary stem-loop structures, pair imperfectly with sites in the 3' untranslated region (UTR) of their target mRNAs, and can apparently act by decreasing target messenger RNA levels or by directly inhibiting translation.^{37–40} In some cases microRNAs have been proposed to act relatively promiscuously and to inhibit multiple targets.^{38,41} Many genes have predicted target sites for several different microRNAs in their 3' untranslated regions, indicating the possibility for combinatorial action of multiple microRNAs to maximize inhibition of gene activity, although for most genes these predictions have not yet been tested. Preliminary *in silico* estimates are that about one-third of all protein-coding genes in the human genome may be regulated in part by microRNAs.^{35,38} The prediction of target genes for particular miRNAs is a difficult computational challenge, and is impaired by the lack of data sets describing alterations in individual protein levels in the proteome in response to alterations in miRNA levels. Target predictions based on mRNA downregulation data are more advanced,

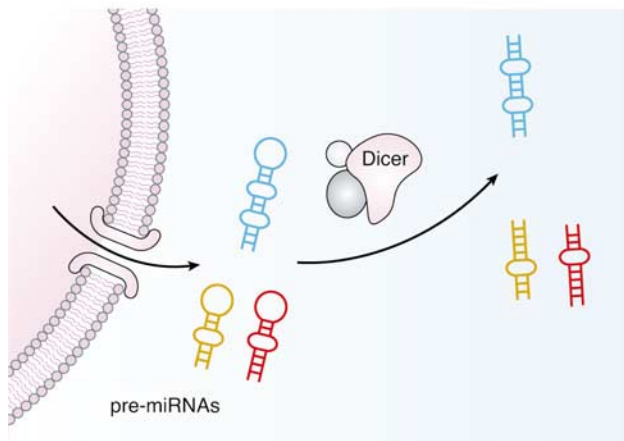
a Pri-miRNAs are transcribed, usually by RNA Polymerase II



b The 'Microprocessor' complex cleaves the pri-miRNAs to form pre-miRNAs



c The Dicer enzyme complex removes the loop from the pre-miRNA hairpin



d One strand, the mature microRNA, is incorporated into the RISC protein complex, and guides translational silencing of the target mRNA

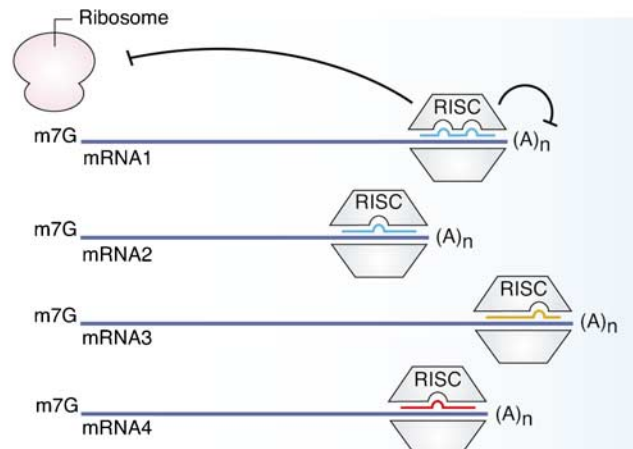


Figure 1 MicroRNA synthesis and function.

due to the greater tractability of mRNA expression profiling. When more protein data are available, it will be interesting to compare the microRNA-target interactions that correlate with effects on target mRNA levels and protein levels.

Biochemical experiments have begun to examine microRNA interactions with their target genes. Cellular subfractionation studies indicate that many miRNAs are found in the polyribosome fraction, which would be most consistent with a role in decreasing productive extension of the polypeptide chain after initiation of translation.^{42,43} Other proposed mechanisms of miRNA action include interference with recognition of the 5' cap of the mRNA to prevent translation initiation, destabilization of mRNA via deadenylation of the poly-A tail, and recruitment of the ribosome-inhibitory protein eIF6.⁴⁴⁻⁴⁷ The detailed mechanisms of miRNA activity, including a full list of the protein components of the active miRISC complex for various miRNAs,

await further elucidation. A recent paper underscores this point with the surprising observation that under some conditions, such as cell cycle arrest, microRNAs can upregulate translation of a target protein.⁴⁸

Evolutionary Considerations

Comparisons between humans and other animals show that many microRNAs are well conserved in evolution, and are likely part of important ancient pathways of gene regulation, while other microRNAs are more restricted in their phylogenetic distribution.^{23,24,49} A comparison of brain-derived microRNAs in humans and chimpanzees uncovered a large number of novel primate- and human-specific microRNAs that have been proposed to relate to the increased complexity and specialization of brain development that occurred in human evolution.¹⁷

Many microRNAs in humans belong to families whose members reside at different chromosomal loci, but are very similar in sequence. Similar to protein-coding gene families with many closely related members, there may be functional redundancy or partial redundancy of these microRNAs. This redundancy will increase the challenge of discovering the microRNA-target relationships and their functional consequences. For example, there are 11 distinct members of the let-7 microRNA family in humans, differing from each other by a few bases at most.⁵⁰

The short length of microRNAs and their binding sites within target genes should give them the opportunity to mutate and change specificity rapidly in evolution. A striking example of the potent effect of such mutation comes from Texel sheep, an exceptionally muscular breed, where a genetic quantitative trait locus has been mapped to a mutated site in the myostatin gene 3'UTR. The mutation creates a new target site for the miR-1 and miR-206 microRNAs which are abundantly expressed in skeletal muscle, causing down-regulation of the skeletal muscle-inhibitory myostatin protein and giving rise to the muscle hypertrophy phenotype.⁵¹ The resultant phenotype is similar to the hyper-muscularity resulting from coding region or splice site mutations in the myostatin gene reported in cattle, mice, dogs, and humans.^{52,53} In humans, some evidence for a similar story has emerged for a hypertension-associated allele of the angiotensin receptor *AGTR1* gene: the 1166C allele disrupts a miR-155 target site in the 3'UTR of the gene, potentially preventing downregulation of the *AGTR1* protein.⁵⁴

Overall, it appears that there is a relative depletion of predicted target sites for microRNAs in the 3' UTRs of messenger RNAs that are coexpressed in the same cells or tissues as the microRNAs, indicating that microRNA-based selective pressures may have a substantial effect on the evolution of potential target genes.⁵⁵ In a different arena of selective pressure, the capacity for rapid evolutionary change in microRNA genes and their target sites has been hypothesized as providing an advantage to animals in responding to rapidly evolving parasites and pathogens.⁵⁶

MicroRNAs IN DEVELOPMENT AND PHYSIOLOGY

Profiles of miRNAs in Normal Human Tissues and Cells

Cloning and measurement of microRNAs from various human tissues has proceeded quickly. We now have an overview of the sites of expression of many microRNAs.⁵⁷ About one-third of microRNAs show substantial tissue specificity, while the others may vary in expression level but are not particularly tissue or cell type-specific, at least at the resolution of currently available data. Among the most specific miRNA expression patterns are that of miR-122 in the liver, miR-375 in pancreatic islet tissue, miR-142 and miR-223 in the hematopoietic system, and miR-1 and miR-133 in muscle.⁵⁷⁻⁵⁹ The utility of some tissue-specific microRNAs for identifying the tissue of origin of poorly differentiated metastases of unknown primary tumors in human patients has been shown

in a proof-of-concept analysis.⁶⁰ Reports of the phenotypes of knockout mice for different microRNA genes and tissue-specific Dicer mutants have begun to appear in the literature, giving valuable insights into the cellular processes that become derailed when particular microRNAs are absent. A cautionary note concerning the overall importance for microRNAs in animal development may be sounded by the results of Dicer mutation in zebrafish, where many cell lineages and embryologic structures developed adequately in the absence of known microRNA pathways.⁶¹ It may be that some animal tissues and cell lineages will be more dependent on microRNA regulation than others.

Stem Cell Biology

A small number of microRNAs are expressed mainly in embryonic stem (ES) cells, and attention has naturally focused on their roles in maintaining the pluripotent undifferentiated state, self-renewal, and other characteristics of ES cells.^{62,63} Some evidence for such roles comes from the poor proliferation and differentiation potential of Dicer-null murine ES cells, and from the small number of germline stem cells produced in *Drosophila* mutants of *Dicer1* or its accompanying RNA-binding protein, *Loquacious*.⁶⁴⁻⁶⁷ In contrast, mouse ES cells null for the microprocessor complex member *DGCR8*, which is also required for microRNA biogenesis, showed a less severe impairment of division in culture while still being unable to differentiate normally into other cell types when appropriately stimulated.⁶⁸ Together, the mammalian ES cell studies of *Dicer* and *DGCR8* mutants show these proteins having overlapping but not identical functions, suggesting that one or both may be involved in other pathways besides the microRNA pathway as it is currently understood. Targeted disruption of ES cell-specific microRNAs can be expected to shed further light on exactly which miRNA effectors may contribute to the observed phenotypes.

Germline

Tissue-specific ablation of the *Dicer* gene in mouse oocytes results in abnormalities culminating in meiosis I arrest with severe defects in meiotic spindle formation and chromosome organization. In addition, mRNAs that are normally degraded in meiotic maturation in oocytes, and that have predicted target sites for oocyte-expressed microRNAs, were present at elevated levels in the *Dicer*-null oocytes, suggesting that the microRNAs may participate in specifying their destruction.⁶⁹ This is reminiscent of the situation shortly after fertilization in zebrafish zygotes, where a single microRNA, miR-430 is postulated to destabilize hundreds of maternally-expressed mRNAs that would otherwise linger in the zygote. In support of this, zebrafish *Dicer* mutants have increased zygotic levels of mRNAs with predicted miR-430 target sites.⁷⁰

Skeletal and Cardiac Muscle

The human and mouse genomes each contain two bicistronic loci, each of which expresses miR-1 and miR-133 microRNAs in skeletal and cardiac muscle.^{71,72} Selective disruption of the miR-1 gene (miR-1-2) from one of the two loci in mice revealed that the absence of this microRNA causes 50% embryonic lethality with frequent ventricular septal defects. Among miR-1-2 knockout mice who survived to adulthood, abnormal cardiac electrical conduction and hyperplasia of cardiac myocytes was observed, despite grossly normal cardiac anatomy and function.⁷³ The results from this mouse strain, which, it should be emphasized, only inactivates one of the two miR-1 microRNAs known to be expressed in cardiac muscle, may indicate an important role for miR-1-2 in the embryological development of the heart, and a lesser role in normal adult cardiac tissue. Of course, the degree of compensation provided by the other miR-1 locus will need to be examined to get a deeper understanding of the role of this microRNA family in cardiac development and maintenance. In another recent study, interference with the muscle-specific miR-133 family members using antagomir (essentially, antisense oligonucleotide) techniques caused the development of cardiac hypertrophy in mice, indicating that the normal role of this cardiac microRNA may be to prevent undesirable myocyte hypertrophy.⁷⁴

Hematopoiesis and Immunity

Normal hematopoiesis and immunity are guided by microRNA-mediated gene regulation. miR-181 is expressed in hematopoietic tissues, and at lower levels in several other tissues such as muscle.⁷⁵ Overexpression of miR-181 in murine hematopoietic progenitor cells increases the relative proportion of B cells to T cells in the peripheral blood; interpretation of the mechanism of this effect is complicated by the role played by this microRNA in modulating thymocyte T cell receptor signalling and clonal selection, but it clearly influences the final balance of the two major lymphoid lineages.^{76–78}

Immunological studies in mice have shown that miR-155 is expressed in germinal center B cells, and is required for normal germinal center formation, antibody titers in response to antigen, and plasma cell differentiation or persistence.^{79–81} Normal functioning of T lymphocytes and dendritic cells also appears to depend on this microRNA.^{79,80} In the absence of miR-155, helper T cells deviate toward the T_H2 phenotype, producing increased levels of IL-4 and less interferon gamma than controls.^{79,80}

In both mouse and human cells, the earliest stages of erythroid cell differentiation are accompanied by dramatic upregulation of miR-451.^{82,83} Indeed, even peripheral red blood cell preparations show significant amounts of miR-451, perhaps indicating a role for this microRNA in the final regulation of mRNA in reticulocytes.⁸⁴

Nervous System

miR-9, miR-124, and miR-128 are among the microRNAs most highly and specifically expressed in the mammalian brain.^{85,86} Deletion of the well-conserved miR-9 in fruit flies causes duplication of sensory neurons and sensory organs in development.⁸⁷ The effects of overexpression or temporarily knocking down miR-124 in chick neural tubes, in contrast, are somewhat controversial, and show, at most, a modest impact of this microRNA on neuronal differentiation.^{88,89} Neuronal differentiation in neuroblastoma and embryonal carcinoma cell lines *in vitro* is enhanced by miR-124, at least in part due to miR-124 inhibiting expression of a regulator of alternate mRNA splicing, PTBP1.⁹⁰ Given the apparent rich diversity of microRNAs expressed at low levels in the mammalian CNS, subtle phenotypes or highly cell type-specific microRNA roles may be the rule in this organ.¹⁷ A hint of these possibilities comes from an example in the nematode worm *C. elegans*, where left–right asymmetry of chemosensory receptor expression in some neurons is controlled by a microRNA named *lsey-6*.⁹¹

MICRORNA IN DISEASE STATES

Given their important roles in normal physiology, it is no surprise that microRNAs have come under close scrutiny in a variety of diseases. MicroRNAs have been shown to play contributory roles in cancer, heart disease, infectious disease, and other medical conditions.

Cancer

Some miRNAs appear to act as oncogenes, by contributing to the transformed phenotype when expressed at elevated levels in cancers. miR-21 is relatively overexpressed in glioblastoma multiforme, cervical cancer, breast cancer, and many other solid tumor types.^{92–94} Increased expression of miR-21 appears to contribute to decreased apoptosis in the malignant cells.⁹³ Similarly, the locus containing seven microRNAs of the miR-17-92 polycistron cluster at human chromosome 13q31 is amplified in a variety of B cell lymphomas, nasal-type NK/T cell lymphoma, and solid tumors.⁹⁵ Overexpression of a portion of this polycistron, miR-17-19b, in a mouse lymphoma model where the *c-myc* gene is regulated by the immunoglobulin heavy chain enhancer, showed cooperation between miR-17-19b and *c-myc* in oncogenesis, causing more rapid lymphoma growth and shorter animal survival.⁹⁶ The targets and mechanisms of miR-17-92 action have yet to be elucidated, but *Myc*-regulated expression of this polycistron also appears to determine the angiogenic potential of colon adenocarcinomas in a different mouse model system.⁹⁷

Conversely, some microRNAs can act as tumor suppressors, genes whose deletion or mutation helps cells along the multi-step process of tumorigenesis. Circumstantial evidence for this possibility first emerged in early microRNA profiling experiments where tumor samples appeared to have lower overall levels of microRNA expression than normal tissue

samples, both in human clinical material and tissues from murine cancer models.⁶⁰ There is some debate at present concerning the extent of global miRNA suppression in human cancers; some of the differences in reported findings may relate to choices of normal samples for comparison, different analytic platforms, a lack of internal standards for each analyte measured, or the use of normalization procedures that could minimize actual global differences between tumor and normal samples.^{60,92}

Some evidence supporting a role for global miRNA suppression in cancer cells came from a relatively small series of non-small cell lung cancer samples that showed decreased survival of patients whose tumors had lower levels of Dicer mRNA expression.⁹⁸ A helpful recent study used RNA interference methods to globally 'knock down' mature microRNA levels by inhibiting expression of Dicer1, Drosha, or DGCR8.⁹⁹ Impaired miRNA pathways led to increased growth of mouse and human cancer cell lines in soft agar cultures, and more malignant behavior when cells were injected into nude mice. Deletion of one or both copies of the Dicer1 gene was also shown to lead to greater tumor burden in a Kras point-mutant mouse model of lung cancer, further supporting the idea that small RNA pathways dependent on Dicer tend to act as an overall brake on cancer growth.⁹⁹ Overexpression of let-7g reversed the *in vitro* effects of Dicer1 knockdown, indicating that let-7 family members may be key tumor suppressors among microRNAs. These findings fit well with earlier mechanistic studies of the let-7 miRNA family that identified Ras genes as targets of inhibition by let-7 miRNAs in both nematodes and human cancer cells.¹⁰⁰

Other examples of miRNA involvement in cancer are suggestive but less easily classified. An initial report described overexpression of miR-155 as a hallmark of pediatric Burkitt's lymphoma, but a subsequent study found that miR-155 was only overexpressed in a minor subset of Burkitt's lymphoma cells, those exhibiting Epstein-Barr virus latency pattern III.^{101,102} Studies of the 13q14 locus commonly deleted in chronic lymphocytic leukemia (CLL) indicated that miR-15a and miR-16-1, the two microRNAs at the locus, could act as tumor suppressors, perhaps by downregulating the antiapoptotic gene *BCL2*.¹⁰³⁻¹⁰⁵ The New Zealand Black mouse model for CLL has also been shown to have a sequence variant in the miR-16 gene *Mir16-1* that causes a modest decrease in levels of miR-16. Attempts to use the overall profile of microRNA expression to predict patient outcome in CLL have identified correlations, but these did not provide extra information beyond conventional prognostic markers such as *IgV_H* gene hypermutation.¹⁰⁴ More recent work measuring miRNA expression in CLL using direct miRNA cloning and quantitative RT-PCR methods instead of microarray hybridization found no correlation between miR-15a or miR-16-1 expression and *Bcl2* levels.¹⁰⁶ Hopefully, further study can clarify the reasons for these different results, and perhaps give further insight into the

reasons why the 13q14 deletion is, in fact, a marker for good prognostic in CLL.

MicroRNAs now seem likely to be important downstream target genes of previously identified transcription factors, as well. Four recent studies identified miR-34a as a gene regulated by the tumor suppressor p53 transcription factor. These reports provide evidence that miR-34a is important for p53-mediated apoptosis, and possibly for other p53-mediated responses to cellular stress.¹⁰⁷⁻¹¹⁰ The mechanism of these effects has yet to be established.

Finally, tumor phenotypes associated with extensive disease, such as invasion of surrounding tissues and metastasis, are also under the influence of microRNA controls. A new cellular pathway governing tumor invasiveness and metastasis has been mapped out in a breast cancer model system in which human breast cancer cells are implanted into the mammary fat pads of mice. The transcription factor Twist regulates expression of microRNA miR-10b, which inhibits translation of the homeobox gene *HOXD10*, preventing it from repressing the gene for the GTPase RHOC, which has been shown to be a metastasis-promoting protein in several studies.^{111,112} The net effect of increased expression of miR-10b is increased invasiveness and metastasis of the tumor cells, a finding consistent with increased expression of this microRNA in primary tumors from patients who had metastatic disease.¹¹¹

On the more clinical end of the research spectrum, profiling of microRNAs in many other kinds of cancers is proceeding rapidly, with recent examples from cervical, prostate, pancreatic, and germ cell tumors, among many others.^{92,94,113-118} Characteristic microRNA patterns offer new information that could yield advances in tumor classification and prognosis determination, although the clinical validation of such efforts will require some time.

Heart Disease

Several recent papers have described the contribution of microRNAs to the physiology of cardiac hypertrophy under conditions of stress and heart failure. Exogenous overexpression of some miRNAs that are upregulated in failing hearts is sufficient to cause cardiac myocyte hypertrophy in culture, and transgenic overexpression of miR-195 in a mouse's heart causes frank cardiac hypertrophy.^{119,120} These experiments demonstrate the potent effect of microRNAs in modulating the physiology of complex organs in the whole animal. In contrast, knocking out another miRNA with a cardiac expression pattern, miR-208, showed that it is necessary for the cardiac hypertrophy and upregulation of beta-myosin heavy chain that occurs in response to increased cardiac afterload.¹¹⁹ The expression of miR-208 was also required for the cardiac upregulation of beta-myosin heavy chain that occurs in the absence of normal thyroid hormone signaling, showing that microRNAs can play roles in the endocrine coordination of cardiac responses.¹¹⁹

Infectious Disease

A number of viruses have long been known to express short non-coding transcripts from their genomes, and it now seems that some short non-coding viral RNAs may make use of, or subvert, the machinery of the microRNA pathway in the host cell. The large γ herpesvirus Epstein–Barr virus appears to encode two clusters of microRNAs, one cluster in the introns of the BART gene, another in the 3' and 5' untranslated regions of the BHRF1 gene.^{121–123} The numerous predicted targets for these viral miRNAs include genes involved in cell proliferation, apoptosis, and immune signaling.¹²³ Similarly, other herpesviruses including Kaposi's sarcoma virus, cytomegalovirus, as well as the SV40 DNA tumor virus, have all been reported to express microRNAs from their genomes.^{122,124–128} The single SV40 miRNA is necessary for regulating the levels of early genes in the viral life-cycle, and contributes to virally-infected cell resistance to cytotoxic T cell lysis.¹²⁶ In contrast, initial analyses of some viruses with RNA genomes, such as yellow fever virus, hepatitis C virus (HCV), and human immunodeficiency virus 1 (HIV) have so far failed to detect virus-encoded microRNAs.¹²² Interestingly, HCV makes use of the host hepatocyte-expressed miR-122 to increase the replication of its own RNA genome.¹²⁹

Some viruses seem to block activity of miRNA pathways altogether. Replication of the primate foamy virus is inhibited by the host cell miR-32, but the virus expresses a transcriptional activator protein, Tas, that can interfere with miRNA activity.¹³⁰ Other inhibitors of RNA-mediated gene silencing pathways have been reported in influenza A, vaccinia, HCV, HIV-1, and Ebola viruses. The specificity of these effects and their role in productive viral infection certainly warrant further investigation.^{131–134}

Efforts are also underway to determine which host cell miRNAs are upregulated in response to individual pathogens. For the moment, miR-155 appears to be a major part of the macrophage inflammatory response, as it is upregulated in murine bone marrow macrophages by Toll-like receptor ligands such as polyriboinosinic: polyribocytosinic acid (poly-I:C), lipopolysaccharide, hypomethylated DNA, and the synthetic lipoprotein Pam3CSK4, as well as interferon beta.¹³⁵ Mice homozygous for an unusual hypomorph allele of *Dicer1* show increased susceptibility to infection by vesicular stomatitis virus (VSV).¹³⁶ VSV mutated to ablate putative binding sites for host miR-24 and miR-93 has increased virulence, suggesting that these host microRNAs could work in a *Dicer1*-dependent manner to limit the course of the viral infection.¹³⁶ In another twist, several cellular microRNAs have been shown to bind HIV-1 encoded transcripts, limiting production of the viral proteins, but ironically contributing to the ability of the retrovirus to remain latent and undetected by the immune system in quiescent CD4-positive T cells.¹³⁷

PRACTICAL ASPECTS

A number of recent methodological papers have provided detailed protocols for measuring microRNA levels in

different kinds of specimens. Real time PCR, microarrays, Luminex bead arrays, Northern blotting, and direct cloning and sequencing approaches have all been applied with success, although there may be some systematic differences between some methods.^{60,138–144} *In situ* hybridization detection of microRNAs has also been reported by several groups.^{145–147} It is noteworthy that standard formalin-fixation and paraffin-embedding of tissues seems to preserve microRNAs in a reasonably intact and extractable state.^{146,148,149} Once these tools are clinically validated more extensively, they should enable microRNA measurements to become a part of the pathologist's diagnostic armamentarium.

CONCLUSIONS

MicroRNAs are important functional threads in the cellular tapestry seen through the pathologist's microscope. Their normal activities help to coordinate multicellular life, and their dysregulation can contribute to a variety of diseases. The functional studies described here are only the first forays into better understanding of microRNA roles. Further studies correlating microRNA activity with mRNA and protein levels in clinically relevant specimens, and more detailed mechanistic studies using cell- and tissue-specific manipulation of microRNA levels in animal models will likely reveal new surprises. Although there is no reason to believe that these small RNA molecules are more important than transcription factors and other protein-encoded gene products in the overall life of the cell, they represent a significant component of the apparatus that regulates gene activity. We can speculate that these small strands will help us to make many meaningful new biological and medical connections in coming years.

NOTE

Space restrictions prevented the discussion or citation of the many other contributions made by numerous labs in this rapidly progressing field. I thank Andrew Fire, the members of the Fire lab at Stanford, and my pathology department clinical colleagues for helpful comments and critical review of the article.

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