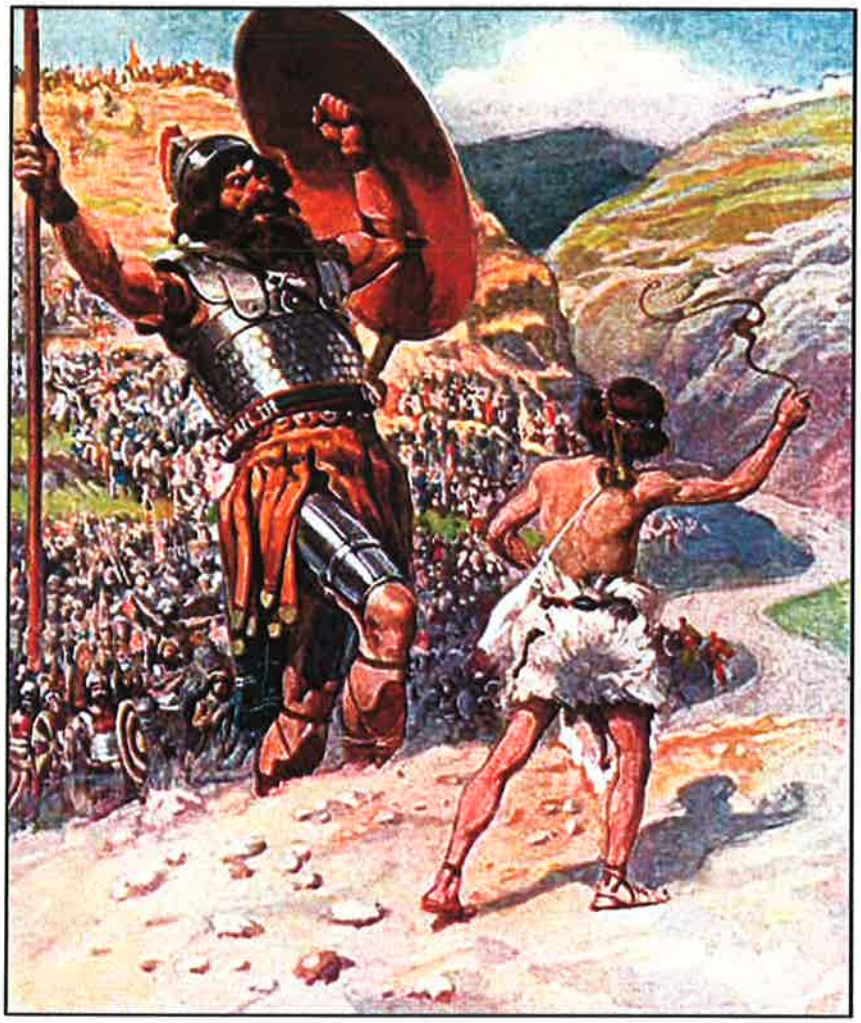
MicroRNA: Bigger Isn't Better!



Peter Igarashi, M.D.

Internal Medicine Grand Rounds

University of Texas Southwestern Medical Center

May 25, 2012

This is to acknowledge that Peter Igarashi, M.D. has not disclosed any financial interests or other relationships with commercial concerns related directly or indirectly to this program. Dr. Igarashi will not be discussing off-label uses in his presentation.

Peter Igarashi, M.D.
Professor of Internal Medicine and Pediatrics
Chief, Division of Nephrology

Dr. Igarashi's interests include kidney development, molecular genetics, and polycystic kidney disease.

Purpose: Review the roles of microRNAs in biology and disease.

Objectives:

1. Understand what microRNAs are and how they function.
2. Learn how microRNAs can be used for diagnosis and prognosis.
3. Learn how microRNAs can be used as targets for therapy.

Cover image: James J. Tissot, *David Slings the Stone* (1896-1900), gouache on board, The Jewish Museum, New York, USA

"David vs. Goliath"

First Samuel, Chapter 17 of the *Old Testament* describes the battle between the Philistines and the Israelites at the Valley of Elah. For 40 days, Goliath, the nine-foot tall champion of the Philistines, comes out between the battle lines and challenges the Israelites to send out a champion of their own to fight him and decide the outcome, but the Israelites are afraid. David, a shepherd boy bringing food for his brothers, accepts the challenge. David and Goliath confront each other, Goliath with his armor and shield, David with only a staff and sling. The Philistine curses David, but David hurls a stone from his sling and hits Goliath in the center of his forehead. Goliath falls to the ground and is killed as the Philistines flee.

The epic battle between David and Goliath is constantly repeated in all cells in our bodies. In this battle, David is represented by microRNAs, short RNA molecules about 20 nucleotides in length. Goliath is represented by messenger RNAs, which are much larger, around 2,000 nucleotides in length or 100-times larger than the microRNAs. Despite the considerable size difference, microRNAs are capable of destroying RNAs.

The purpose of this review is to discuss the roles of microRNAs in biology and human disease. The review is divided into three parts: The first part, entitled "David vs. Goliath", will discuss what microRNAs are and how they work. The second part, entitled "The Goldilocks Principle", will discuss the functions of microRNAs in biology and diseases. The third part, entitled "Back to the Clinic" will discuss clinical aspects of microRNAs.

What are MicroRNAs?

Fig. 1 (left) depicts the **"Central Dogma of Molecular Biology"** as stated by Dr. James Watson. According to the dogma, genetic information is contained in the sequence of DNA. DNA can be replicated, allowing the genetic information to be transmitted to progeny, or the DNA sequence can be copied into an RNA molecule, called messenger RNA (mRNA). The synthesis of RNA from DNA occurs in the nucleus and is referred to as transcription. The mRNA then exits the nucleus into the cytoplasm where it binds to ribosomes that translate the nucleic acid sequence into the amino acid sequence of a protein. In this manner, the genetic information contained in DNA is transferred to RNA and then to protein. In addition to mRNAs that encode proteins, DNA can also be transcribed into RNA species that do not encode proteins, so-called **non-coding RNAs**. As shown in Fig. 1 (right), there are many types of non-coding RNAs, including ribosomal RNAs (rRNAs) that compose ribosomes, transfer RNAs (tRNAs) that are involved in translation, small nuclear RNAs (snRNAs) that are involved in RNA splicing, TERC, the RNA component of telomerase, and microRNAs (miRNAs). The diversity of non-coding RNAs and the fact that some RNAs can function

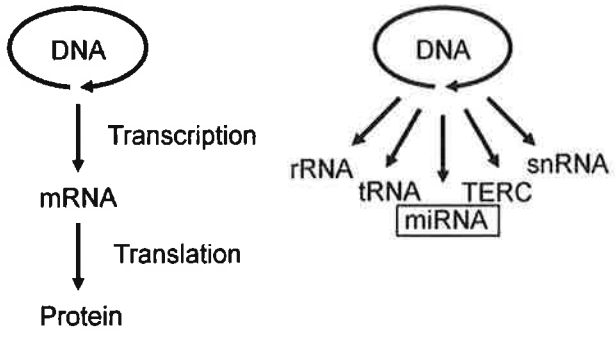


Figure 1. Coding (left) and non-coding RNAs (right)

catalytically, similar to enzymes, has suggested the existence of an "RNA World" that may have preceded the emergence of the current protein world.

MicroRNAs (miRNAs) are short, double-stranded RNA molecules that are typically 19-25 nucleotides in length. miRNAs are too short to encode proteins. Instead, miRNAs play regulatory functions. They bind to specific mRNA targets and inhibit their expression. Since this regulation occurs after the mRNA has already been transcribed, it is a form of **post-transcriptional gene regulation**. As will be seen shortly, miRNAs play important roles in virtually all biological processes and diseases, including development, cancer, viral infection, and tissue fibrosis (1-3).

How do MicroRNAs Work?

Fig. 2 illustrates how miRNAs work. The miRNA associates with a protein complex depicted by the green oval. This protein complex is called the **RNA-induced silencing complex (RISC)** and is comprised of subunits from the Argonaute and Piwi families (4, 5). Once the miRNA binds to the RISC complex, one of the two strands of the miRNA (called the guide strand) recognizes and binds to a complementary sequence in an mRNA target. Usually, miRNAs bind to sites located in the **3' untranslated region of mRNA**. Recall that mRNAs contain a coding sequence, or open-reading frame (ORF), consisting of the nucleic acid sequence that is translated into protein. The coding sequence is flanked by additional sequences that are not translated into proteins, the 5' untranslated region (5'UTR) and 3' untranslated region (3'UTR). In most cases, miRNAs bind to the 3' UTRs of mRNAs, although binding to the 5' UTR has also been reported (6). Once a miRNA binds to its target mRNA, the mRNA is degraded (4). In the case where the sequence of the miRNA is perfectly or nearly complementary to the mRNA sequence, the RISC complex cleaves the target mRNA and destroys it. This process is rare in mammals, where more commonly the miRNA is partially complementary to the mRNA. In this latter case, the RISC complex can either inhibit protein translation or promote mRNA degradation (7). The exact molecular mechanisms are still being elucidated, but degradation first involves deadenylation (removal of the polyA tail) followed by digestion by exonucleases (8). In some cases, the targeted mRNA may be sequestered in subcellular organelles called P bodies where it is not available for translation (9). Recent studies suggest that in mammals miRNAs primarily function by promoting mRNA degradation (10). Regardless of the exact mechanism, the result is that the expression of the protein encoded by the mRNA is inhibited. However, it should be noted that in a small number of cases, miRNAs can also stimulate gene expression (11).

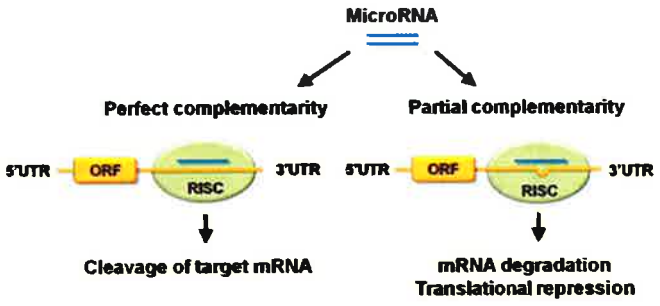


Figure 2. microRNAs bind to mRNA and inhibit post-transcriptional gene expression

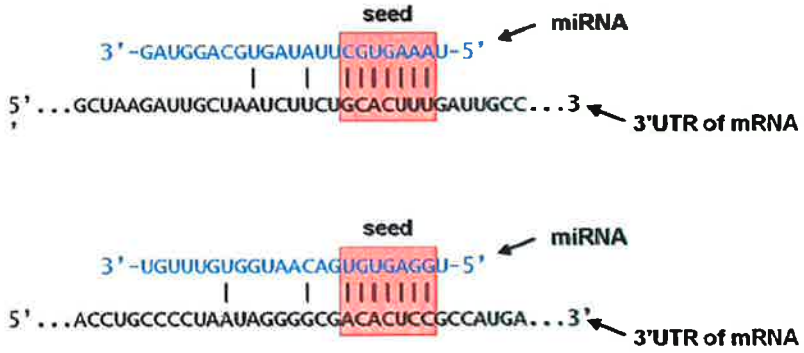


Figure 3. MicroRNAs bind to the 3' untranslated regions of mRNA

Fig. 3 shows a higher magnification view of the interaction between two miRNAs and their respective mRNA targets. The guide strands of the miRNAs are shown in blue. The 3'UTRs of the mRNA targets are shown in black. miRNAs recognize and bind to complementary sequences in mRNA by Watson-Crick base pairing (vertical lines). The red boxes depict the so-called **"seed sequence"** that is located at the 5' end of the miRNA, from nucleotides 2-8. The seed sequence is required for binding of an miRNA to its target (12). Different miRNAs may contain the same or different seed sequences. miRNAs that share the same seed sequence usually recognize the same mRNA targets, whereas miRNAs that have different seed sequences, as shown in Fig. 3, recognize different mRNA targets (unless both seed sequences happen to be present in the same mRNA). Outside of the seed sequence, the sequence of the miRNA does not need to be complementary to the target mRNA, although such complementarity may enhance binding. Since the sequences of all protein-coding genes have now been determined, it is possible to predict the genes that may be regulated by a particular miRNA based on complementarity to the seed sequence (12). Several software programs exist for this purpose (TargetScan, PicTar, miRanda, etc.), although identifying and validating the actual mRNA targets of a particular miRNA remains a daunting task.

Biosynthesis of MicroRNAs

Fig. 4 shows the biogenesis of microRNAs (4, 9). Like other non-coding RNAs, miRNAs are transcribed from genomic DNA in the nucleus. The genetic loci that encode miRNAs may be located in the DNA between genes (intergenic) or within genes themselves, typically in the introns. In the first case, the expression of a miRNA is usually regulated by its own promoter. In the second case, the expression of the miRNA is regulated by the promoter of the host gene. This can produce interesting interactions between gene transcription and post-transcriptional gene regulation. In either case, the miRNA is first transcribed as a long primary miRNA (pri-miRNA), which is then processed by an enzyme called **Drosha** to produce a precursor miRNA (pre-miRNA)

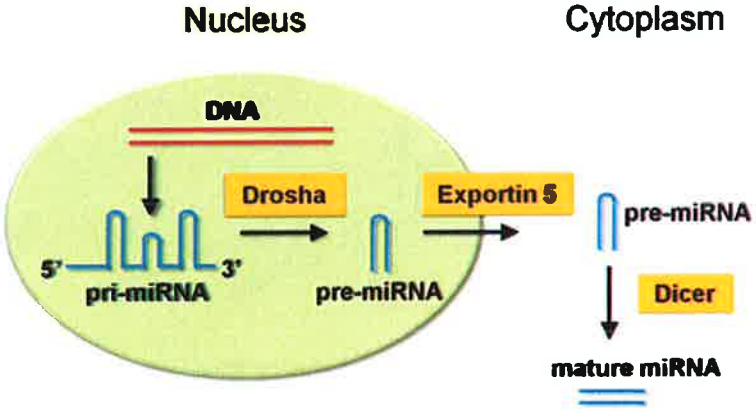


Figure 4. Biosynthesis of microRNAs

that is typically 50-70 nucleotides in length. As shown in the figure, the pre-miRNA forms a hairpin structure because of intramolecular base pairing. The pre-miRNA then exits the nucleus via exportin 5. In the cytoplasm, the pre-miRNA is cleaved by a second enzyme called **Dicer** to produce the mature miRNA, which will then be loaded onto the RISC complex to seek and destroy its mRNA targets. In addition to the canonical miRNA biogenesis pathway shown in Fig. 4, miRNAs can also be produced via alternative pathways. For example, some microRNAs bypass the Drosha step and are synthesized by the RNA splicing machinery instead (so-called miRtrons).

MicroRNAs were first discovered in the roundworm, *Caenorhabditis elegans* (*C. elegans*) (13). In 1993, two groups led by Victor Ambros and Gary Ruvkun reported the cloning of a gene called ***lin-4*** that was required for developmental timing in *C. elegans* (14, 15). Curiously, when the *lin-4* gene was sequenced, the investigators were unable to identify an open-reading frame, so they concluded that *lin-4* was a non-coding gene. The discovery of *lin-4* went largely unnoticed outside the worm community until 1998, when Andrew Fire and Craig Mello discovered **RNA interference**, also in *C. elegans (16)*. They performed an elegant experiment in which short RNA molecules were introduced into worms and silenced the expression of the complementary mRNAs. Double-stranded RNA was required as the single strands by themselves had no effect. Fire and Mello called this phenomenon RNA interference, and we now know that the same process occurs in most multicellular organisms, including plants. For their discovery of RNA interference, Fire and Mello received the 2006 Nobel Prize for Physiology or Medicine.

MicroRNA Complexity

The year 2000 was a watershed year for miRNAs. In that year, the second miRNA was cloned, called ***let-7*** (17). In contrast to *lin-4*, which is only expressed in *C. elegans*, *let-7* was identified in worms, flies, and humans (18). Importantly, the 21-nucleotide sequence of *let-7* is absolutely conserved between these organisms, which span almost one billion years of evolutionary time. Only the sequence of the mature *let-7* miRNA is conserved; the rest of the pre-miRNA is not. This striking degree of conservation suggested immense importance of this short RNA sequence. The following year, it was recognized that RNA interference and miRNAs work through the same mechanism and that Dicer is required for both processes (19). In addition, it was found that mammals

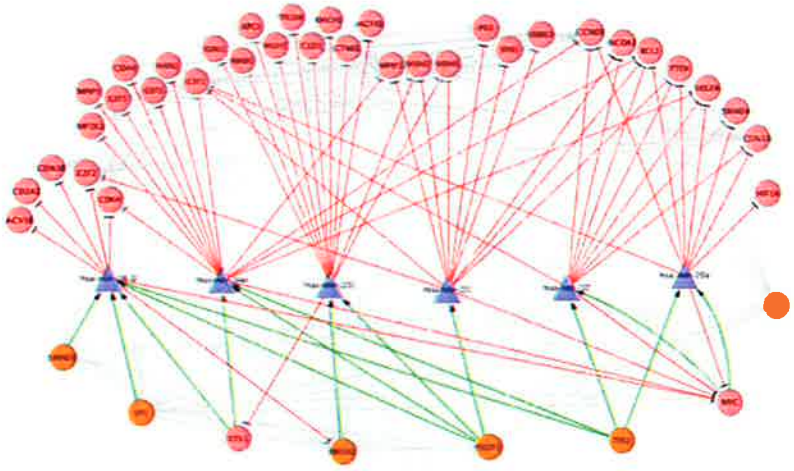


Figure 5. MicroRNA network in ovarian cancer. Blue indicates miRNAs. Red indicates target genes. Orange indicates transcription factors that regulate miRNA expression. Ref: Schmeier S, et al. BMC Syst Biol, 2011

express many different miRNAs in addition to *let*-7 (20-22). Since 2001, work on miRNAs has accelerated rapidly. To date, there are 16,332 publications on microRNAs in Pubmed. The number of miRNAs that have been identified has also grown considerably, to 1,527 in humans (miRBase 18), although less than a third have been validated. About half of the miRNAs reside in intergenic regions, and half reside within host genes. Another property that has emerged is that miRNAs are sometimes present in clusters containing 2-6 miRNAs whose expression is co-regulated. Usually the members of a cluster have similar or identical seed sequences.

In addition to the large number of miRNAs in the genome, each miRNA can bind to multiple different mRNA transcripts and regulate their expression. The 7-nucleotide seed sequence of a particular miRNA will occur by chance once every 4^7 nucleotides (=16,384 nt) in the genome. Since, the human genome contains ~20,500 protein coding genes with an average 3'UTR length of 770 nucleotides (Human Genome Project), it can be estimated that each miRNA could target as many as 960 mRNA transcripts. Since binding affinity is influenced by sequences that are outside the seed sequence, the actual number of high-affinity targets will be less, but is still estimated in the hundreds. This means that individual miRNAs can regulate the levels of expression of hundreds of genes. Moreover, each mRNA transcript can contain binding sites for multiple different miRNAs as well as multiple binding sites for the same miRNA. It has been estimated that more than one-third of human genes are regulated by miRNAs (23). This situation leads to an almost overwhelming degree of complexity involving networks of multiple miRNAs working in concert or opposition to regulate the expression of thousands of mRNA transcripts (**Fig. 5**) (24). However, it is precisely this degree of complexity that allows miRNAs to regulate and coordinate the expression of multiple genes that may be involved in a particular biological response or pathway.

"The Goldilocks Principle"

"Goldilocks and the Three Bears" is a children's fairy tale that was first published by the poet Robert Sotheby in 1837 in a book ironically named "The Doctor." The original story has been revised several times, and in its current form reads as follows: "Once upon a time there were Three Bears who lived in the woods, a great, huge Papa Bear, a middle-size Mama Bear, and a little, wee Baby Bear. One day, after they had made the porridge for their breakfast, the three bears walked out into the woods while their porridge was cooling. And while they were walking, a little girl named Goldilocks came to the house, peered in, and saw the porridge on the table. First she tasted the porridge of the Papa Bear, and that was too hot. And then she tasted the porridge of the Mama Bear, and that was too cold. And then she tasted the porridge of the Baby Bear, and that was neither

"The Three Bears" Arthur Rackham (1867-1939)

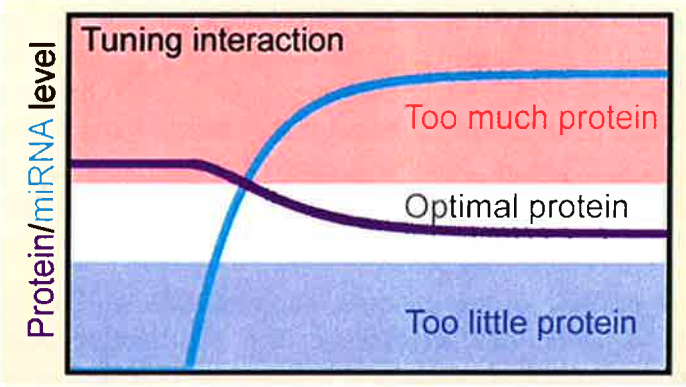


Figure 6. MicroRNAs fine-tune gene expression. Ref: Bartel DP, Cell, 2009.

too hot nor too cold, but just right; and she liked it so well that she ate it all up."

This tale forms the basis for "The Goldilocks Principle", which describes any situation where something must fall within a certain range, as opposed to reaching extremes. The "Goldilocks Principles" has been applied to astronomy to refer to planets that are neither too close nor too far from a star to support life. Applied to economics, it refers to an economy that sustains moderate growth and low inflation. The principle has even been used to describe Obama's foreign policy, neither too hot nor too cold.

Normal Functions of MicroRNAs

In biology, the Goldilocks Principle perfectly describes the function of microRNAs. **Fig. 6** depicts the temporal relationship between a microRNA and its target protein (12). When the level of the protein exceeds the optimal physiological range, the expression of the miRNA is induced. As the expression of the miRNA rises, the expression of the mRNA encoding the protein is inhibited, and the level of the protein falls into the optimal range. In this manner, miRNAs function to maintain the levels of proteins within a narrow range, neither too high nor too low. One additional point that this figure makes is that the changes in protein expression that are produced by miRNAs are relatively modest (25). This turns out to be a general property of miRNAs and explains how they are able to finely regulate gene expression (26).

One of the most elegant examples of miRNA function can be found in the organism in which they were originally discovered, *C. elegans* (17). During development. *C. elegans* progress through four larval stages before reaching adulthood. The entire process from fertilization to adulthood takes about two days. Each larval stage is separated by a molt and involves changes in gene and protein expression. The transitions between larval stages are regulated by miRNAs (**Fig. 7**). The first miRNA that was discovered, *lin-4*, is essential for the transition from the L1 stage to the L2 stage. *Lin-4*

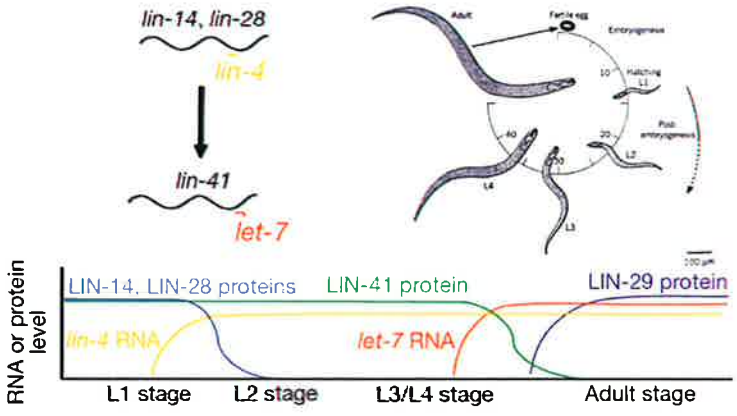


Figure 7. MicroRNAs regulate developmental timing in *C. elegans*. Adapted from Reinhart, Nature, 2000.

binds to two RNA transcripts, *lin-14* and *lin-28*, and down-regulates their proteins. The down-regulation of the LIN-14 and LIN-28 proteins is required for the transition from L1 stage to the L2 stage. Worms that lack the *lin-4* gene fail to make this transition correctly and continue to express early larval stage proteins later in development. Conversely, overexpression of *lin-4* results in premature differentiation. Later in development, a second microRNA, *let-7*, is required for the transition from late larval stages to adulthood. *Let-7* binds to the *lin-41* RNA and down-regulates the expression of the LIN-41 protein. LIN-41 is a transcriptional repressor. When its expression is inhibited, the expression of LIN-29 is increased, which allows the transition to adulthood. These studies exemplify the crucial role that miRNAs play in development.

Over the past decade, it has been recognized that microRNAs play important roles in virtually all biological processes in mammals. In addition to embryonic development, miRNAs regulate hematopoiesis, cell differentiation, proliferation, cell death, angiogenesis, immune response, stem cell division, and lipid metabolism (27-31). MicroRNAs are frequently involved in maintaining normal tissue homeostasis. Given their important roles in normal physiology, it is not surprising that abnormalities of microRNAs are frequently observed in human diseases. Not only is abnormal miRNA expression a marker of disease, but miRNAs can also play significant roles in disease pathogenesis. As one example, we will now discuss the roles of miRNAs in cancer (2, 32-34).

Deregulation of MicroRNAs in Cancer

In a seminal study published in PNAS in 2002, Calin et al. discovered that the expression of two microRNAs, miR-15a and miR-16, is frequently lost in chronic lymphocytic leukemia (CLL) (35). miR-15a and miR-16 are located precisely within a chromosomal region (13q14) that is frequently deleted in patients with CLL. miR-15a and miR-16 regulate the expression of the oncogene Bcl2 (36). Therefore, when miR-15a and miR-16 are deleted, the expression of Bcl2 increases, which promotes cell survival and tumorigenesis. The significance of these miRNAs is elegantly illustrated by studies in which miR-15a and miR-16 are knocked out in mice (37). The knockout mice develop a B-cell lymphoproliferative disorder, which verifies that miR-15a and miR-16 can function as **tumor suppressors** (**Table 1**). Other miRNAs that

Table 1. Roles of miRNAs in cancer

MicroRNA	Function	Cancer type	Target genes
miR-17~92	Oncogene	Lymphoma, lung, breast, stomach, colon, pancreatic, neuroblastoma	PTEN, BIM, CDKN1A, DKK3, ZBTB4
miR-155	Oncogene	Chronic lymphocytic leukemia, lymphoma, lung, breast, colon	SHIP1, SOCS1, CEBPB
miR-21	Oncogene	Many	PTEN, PDCD4, TPM1
miR-15/16-1	Tumor suppressor	Chronic lymphocytic leukemia, prostate	BCL2, MCL1, CDK6, CDC27, CCND1, WNT3A
let-7	Tumor suppressor	Lung, colon, gastric, ovarian, breast	KRAS, MYC, HMGA2
miR-26a	Tumor suppressor	Hepatocellular carcinoma, lung, lymphoma, nasopharyngeal	CCND2, CCNE2, EZH2

function as tumor suppressors include *let-7*, the first miRNA that was discovered in humans. *Let-7* inhibits the expression of the Ras oncogene, and overexpression of *let-7* inhibits tumor growth in a mouse model of non-small cell lung cancer (38, 39).

MicroRNAs can also function as **oncogenes** (**Table 1**). The first example of this type that was discovered is miR-17~92, a cluster of six miRNAs located on Chr 13. Upregulation of miR-17~92 is observed in many human cancers, which has led to the designation of miR-17~92 as an OncomiR. Overexpression of microRNAs may result from amplification of DNA sequences encoding miRNAs or over-activity of transcription factors, such as c-Myc, that regulate miRNA transcription (40). The upregulation of miRNAs may be important in cancer pathogenesis. For example, overexpression of miR-17~92 accelerates tumor development in a mouse model of B-cell lymphoma, suggesting that miR-17~92 can function as an oncogene (41). Among the genes that are regulated by miR-17~92 is the tumor suppressor PTEN. These findings indicate that miR-17~92 may function as an oncogene by inhibiting the expression of tumor suppressors. The overexpression of miR-17 can be inhibited using antagomirs (see below). Administration of miR-17 antagomirs to mice that have been implanted with neuroblastoma cells prevents tumor growth, suggesting a possible future therapy (42). Another miRNA that can function as an oncogene is miR-155. Upregulation of miR-155 was first found in B-cell malignancies, and transgenic overexpression of miR-155 in mice also produces lymphoma (43).

MicroRNAs have also been implicated in **cancer metastases** (44). Increased expression of miR-10b has been linked to breast cancer metastasis (**Fig. 8**) (45). The mechanism appears to involve inhibition of the transcription factor HoxD10 by miR-10b. HoxD10 inhibits the expression of RhoC, a small GTPase that regulates the actin cytoskeleton. In this manner, overexpression of miR-10b leads to overexpression of RhoC and promotes tumor cell migration and invasion. Remarkably, therapeutic silencing of miR-10b using anti-miRs suppressed the formation of lung metastases in a mouse model of breast cancer (46). Another example is miR-200, a microRNA that is expressed exclusively in epithelial cells. Loss of miR-200 results in upregulation of its targets ZEB1 and ZEB2, which promote epithelial-mesenchymal transition (EMT) and tumor metastases (47, 48).

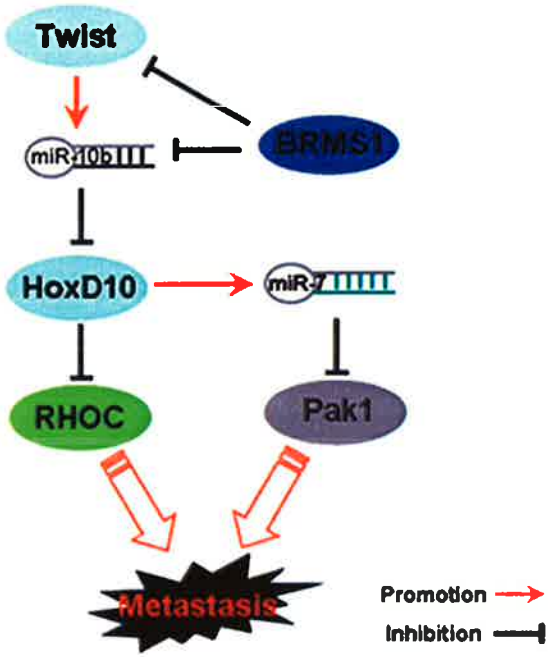


Figure 8. Roles of miRNAs in cancer metastases. Ref: Zhang H et al, Oncogene, 2010

In addition to cancer, microRNAs have now been implicated in virtually all human diseases affecting numerous organ systems (49-55). Examples are shown in **Table 2**.

Table 2. Deregulation of miRNAs in diseases

Disease	miRNAs
Diabetic nephropathy	miR-192
COPD	miR-146a, *let*-7c, miR-125b
Cardiac hypertrophy	miR-23a, miR-208, miR-133
Tissue fibrosis	miR-29, miR-21
Myocardial ischemia	miR-15
Fatty liver disease	miR-241, miR-146b, miR-122
Diabetes	miR-15a, miR-28, miR-126, miR-223, miR-320
Psoriasis	miR-203
Sickle cell disease	miR-320
Tuberculosis infection	miR-144*
Systemic lupus erythematosus	miR-155
Rheumatoid arthritis	miR-155, miR-146a

"Back to the Clinic"

This chapter will review clinical aspects of microRNAs. Although the focus will be on cancer, where many of the original studies have been performed, the concepts can be generalized to many diseases of interest. First, we will discuss the utility of miRNAs in diagnosis, prognosis, and as non-invasive biomarkers. Next, we will review the results of the first clinical trial of a microRNA-targeted therapy in humans.

miRNAs and Diagnosis

Based on the early observations of deregulated expression of specific miRNAs in CLL, investigators have explored the utility of miRNAs for diagnosis of other types of cancer. During this time, methods became available for measuring the expression of multiple different miRNAs simultaneously, initially using microarrays and later deep sequencing. This approach is analogous to the methods that had previously been used for mRNA profiling in cancer. In one of the first studies of this type, Lu et al measured the expression of 217 miRNAs in colon, kidney, prostate, uterus, lung, and breast cancer and compared the expression with normal tissues (**Fig. 9**) (56). Several important observations were made. First, the **miRNA profiles** of cancers are very different from normal tissue, an observation that has subsequently been borne out for all cancers that have been examined. Some common miRNAs are overexpressed in cancer,

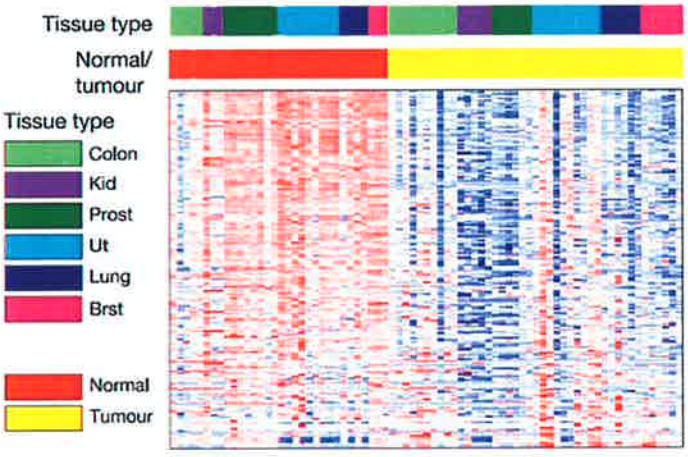


Figure 9. miRNA deregulation in cancer. Heatmap is shown with red indicating higher expression and blue indicating lower expression. Ref: Lu, Nature, 2005

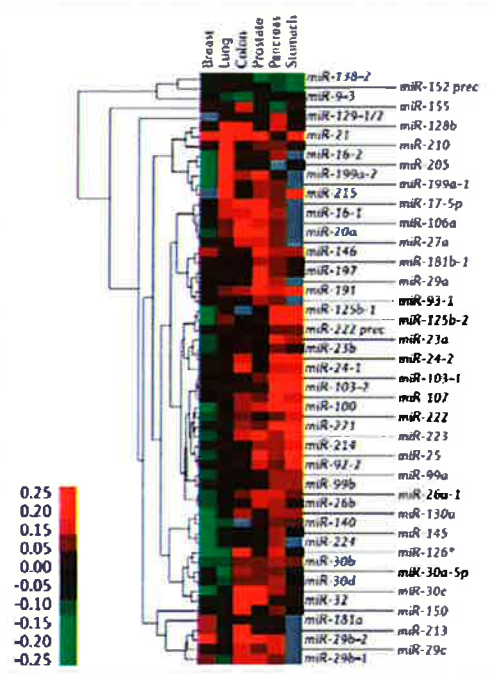


Figure 11. miRNA signatures in cancer. Ref: Volinia, PNAS, 2006

whereas others are down-regulated. In general, Lu et al found that miRNA expression was lower in cancer compared to normal tissue. Second, the pattern of miRNA expression is tissue-specific. That is, some microRNAs are widely expressed but others are only found in certain tissues. This implies that it may be possible to identify a "**miRNA signature**" that identifies a specific cancer (57).

Fig. 10 shows one such example, where upregulation is shown in red and down-regulation is shown in green (58). Each column depicts a different cancer source. As can be seen, each cancer has a distinct pattern of miRNA expression. Therefore, the pattern of miRNA expression can be used to distinguish between different cancers. MicroRNAs have been used to distinguish normal tissue from tumor (59, 60). They have also been used for **histologic classification**, e.g., to distinguish SCLC from NSCLC. Although this distinction might be made easily by histology, in some cases, such as fine-needle aspiration, the material that is obtained may not be conclusive. In this case, miRNA profiling might be helpful in establishing the diagnosis. Another not uncommon clinical problem is the **cancer of unknown primary**. One study by Rosenfeld et al. showed that miRNA profiling may be useful in such cases and can successfully identify the tissue of origin in 89% of cases (61). Compared with conventional mRNA profiling, miRNA profiling often outperforms the older method (56, 61). A related clinical problem is distinguishing primary vs. metastatic disease, e.g., lung nodules. Here, the tissue-specificity of miRNA expression may be useful. Finally, miRNA profiling may be useful for **early diagnosis**. For example, overexpression of miR-205 and miR-21 in pancreatic ductal adenocarcinoma precedes phenotypic changes in the ducts (62). Although the use of miRNA profiling remains in its infancy and is not FDA-approved for any indication, these examples illustrate the great potential of this technology in clinical diagnostics.

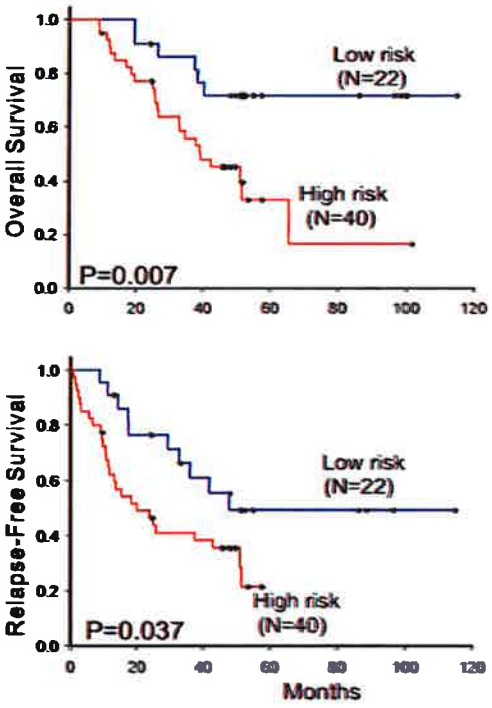


Figure 10. miRNAs predict lung cancer prognosis. Ref: Yu, Cancer Cell, 2008

miRNAs and Prognosis

Several studies have shown that miRNA profiling may be useful for determining the prognosis of disease. In one of the first examples, published in NEJM in 2005, George Calin and colleagues found that a 9-miRNA signature could separate patients with **chronic lymphocytic leukemia** into those with a long vs. short time between diagnosis and start of treatment (63). In one of the best-designed studies of this type, published in Cancer Cell in 2008, a Chinese group used miRNAs to predict prognosis in **non-small cell lung cancer** (NSCLC) (64). First, they identified a 5-miRNA signature based on analysis of tumors from 112 consecutive patients with NSCLC, divided into a training set (n=56) and a testing set (n=56). Two miRNAs were found to be protective (*let-7*a and miR-221) and three conferred high-risk (miR-137, -372, -182). A risk score was calculated based on the expression of these five miRNAs and was tested in an independent validation cohort from a different hospital. **Fig. 11** shows the findings in the validation cohort in which the risk score predicted overall and relapse-free survival. Importantly, the risk score was independent of stage and histology, suggesting that it may be useful for prognosis within cancers with the same histology. **Table 3** shows additional examples in which miRNA profiling has been useful for predicting survival. In addition, several studies have shown that miRNA profiling may be useful for predicting response to therapy. For example, high miR-21 expression is associated with poor response to chemotherapy or molecular therapy in colon and pancreatic cancer (65, 66), whereas low miR-26 expression predicts better response to interferon alfa in hepatocellular carcinoma (67).

Table 3. Prognostic value of miRNAs

Cancer	MicroRNA	Outcomes
NSCLC	*let-7*	Shorter postoperative survival
Lung adenocarcinoma	*let-7*a, miR-155	Shorter survival
NSCLC	5-miRNA signature risk score	Poor overall and disease-free survival
Chronic lymphocytic leukemia	9-miRNA signature	Time to treatment
Gastric cancer	7-miRNA signature	Overall and relapse-free survival
Melanoma	miR-191, miR-193a	Shorter survival time
Colon adenocarcinoma	miR-21	Shorter survival, poor response to therapy
Pancreatic cancer	miR-21	Shorter survival, poor response to gemcitabine
Hepatocellular carcinoma	miR-26	Shorter survival but better response to interferon alfa

miRNAs as Biomarkers

In the examples discussed above, miRNA profiling has been performed on tissues. Recent studies raise the possibility of measuring miRNAs in blood and other **body fluids** as non-invasive biomarkers of human disease (68-70). MicroRNAs have been detected in serum, plasma, urine, saliva, sputum, and CSF. Moreover, the expression of miRNAs in these fluids may correlate with disease states. MicroRNAs have several properties that are ideal for biomarkers, including: high stability, easily assayed by qRT-PCR or other techniques (high sensitivity), and tissue-specific (high specificity).

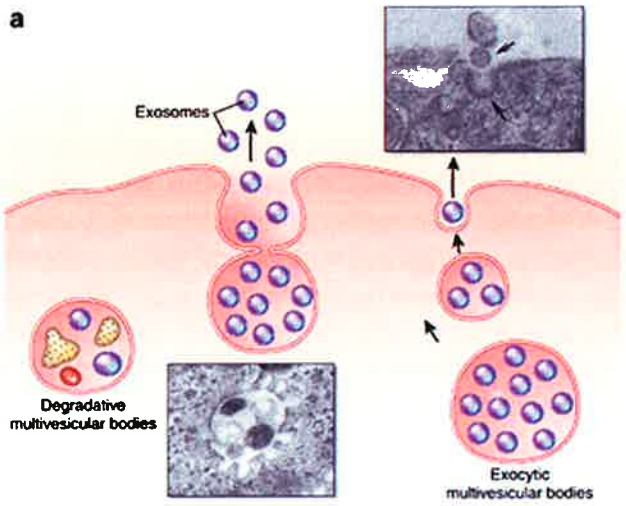


Figure 12. Release of exosomes from the cell surface. Ref: Camussi, Kidney Int, 2010

At first, it was surprising that microRNAs could be detected in blood and other body fluids. Those who have worked with RNA in the laboratory know that it is notoriously susceptibility to degradation. Elaborate precautions must be taken to prevent exposure to ribonucleases that are present on many surfaces, including the skin. Therefore, it was not expected that miRNAs would be present in fluids that contain high ribonuclease activity, such as blood. The explanation is that circulating miRNAs are protected within exosomes (71). **Exosomes** are small (30-100 nm diameter) lipid membrane vesicles that are released by cells into blood, urine, and other fluids. Exosomes are produced within multivesicular bodies (MVB) in the cell, then are released by fusion of the MVB with the cell membrane (**Fig. 12**). Exosomes have been shown to contain proteins, receptors, mRNA, and miRNAs. The function of exosomes in the blood, urine, and other fluids is not clear, but they may function in cell-cell signaling. For example, exosomes produced by cells infected with Epstein-Barr virus can be released and taken up by non-infected cells. The exosomes contain miRNAs encoded by EBV, which then inhibit gene expression in the uninfected recipient cell (72). In this manner, microRNAs may function as hormones!

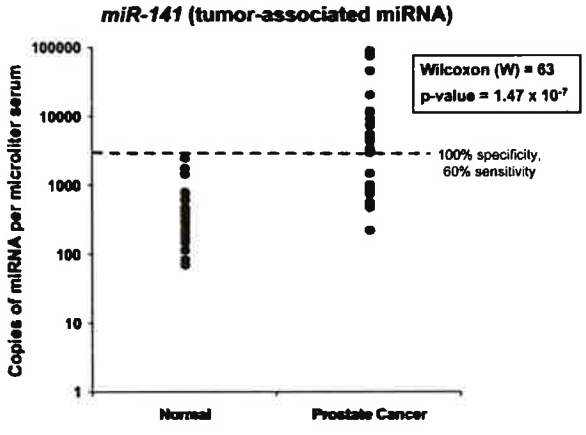


Figure 13. Serum miR-141 is a circulating biomarker of prostate cancer. Ref: Mitchell, PNAS, 2008

Fig. 13 shows one of the first studies using microRNAs as biomarkers published in 2008 (73). Mitchell et al purified the low-molecular weight fraction of RNA from serum from 25 patients with **metastatic prostate cancer** and 25 healthy age-matched male

controls. They measured the expression of six candidate miRNAs by qRT-PCR and found that miR-141 showed the greatest differential expression. Serum levels of miR-141 were 46-fold higher in prostate cancer than controls. The sensitivity o the assay was 60% and specificity was 100%. Receiver operating characteristic (ROC) analysis showed an area under the curve (AUC) of 0.907. miR-141 is an epithelial-specific miRNA that is upregulated in epithelial cancers, including breast, lung, colon, and prostate. These findings establish that tumor-derived miRNAs can serve as circulating biomarkers of common human cancers. Since the initial studies in 2008, many studies have been performed to identify miRNA biomarkers for a variety of human diseases. Examples are shown in **Table 4**.

Table 4. Examples of miRNA biomarkers

Disease	Source	MicroRNA
Myocardial infarction	Plasma	miR-449
Prostate cancer	Serum	miR-141
Bladder cancer	Urine	miR-126/miR-182 ratio
Oral squamous cell carcinoma	Saliva	miR-125a, miR-200a
Acute liver injury	Plasma	miR-122
Lung cancer	Sputum	miR-205, miR-210, miR-708
Renal allograft rejection	Urine	miR-210, miR-10

In the examples listed in **Table 3**, miRNAs are used as biomarkers to identify individuals with established disease. More interesting might be the use of miRNAs to **predict** the future development of disease. In a seminal paper published in PNAS last year, Boeri et al found that plasma miRNA levels may predict those who will develop lung cancer (74). They took advantage of several ongoing studies that are using annual CT scans to screen for lung cancer in heavy smokers. As discussed in Dr. Ron Peshock's grand rounds last week, screening with CT scans is associated with high cumulative radiation exposure. It would be desirable to identify an inexpensive, non-invasive screening method that does not require ionizing radiation. The INT-IEO Trial is a European study in which heavy smokers were followed for four years with annual CT scans and also had blood collected yearly. Boeri et al analyzed the expression of miRNAs in plasma samples that were obtained 1-2 years prior to

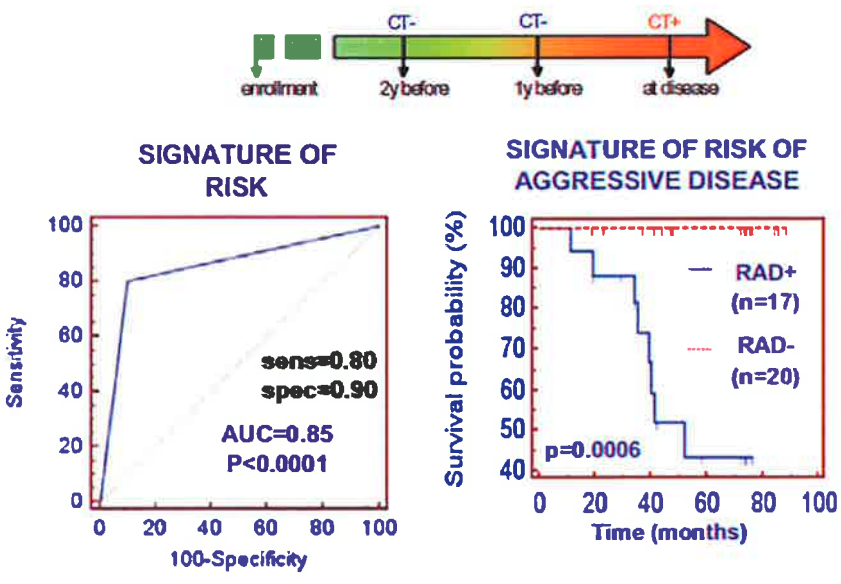


Figure 14. Plasma miRNAs predict the development of lung cancer. Ref: Boeri, PNAS, 2011

the first evidence of lung cancer by CT scanning. They identified 16 miRNA ratios that were increased in patients who were subsequently diagnosed with lung cancer. They then tested the predictive value of the miRNA ratios in an independent lung cancer screening study (MILD). As shown in **Fig. 14**, analysis of miRNA ratios correctly predicted individuals who would subsequently develop lung cancer with a sensitivity of 80%, specificity of 90%, and ROC AUC of 0.85. The predictive value of circulating miRNAs in lung cancer has subsequently been verified by other investigators (75). These promising early studies suggest that circulating miRNA biomarkers may be useful for identifying high-risk individuals who warrant closer observation.

miRNAs as Therapeutics

The observation that miRNAs can play pathogenic roles in diseases has raised hopes that they might be targets for novel molecular therapies. Several approaches have been devised for targeting miRNAs in vivo (**Fig. 15**). In diseases that are associated with down-regulation of miRNAs, the deficient miRNAs may be replaced with **miR-mimics**, which are double-stranded RNA molecules that are identical in sequence to the mature miRNA. Like mature miRNAs, miR-mimics can be loaded on to the RISC complex and degrade target mRNAs. Alternatively, miRNAs may be restored using **viral vectors**. In one such example, Dr. Josh Mendell, who is now on the faculty at UT Southwestern, used adeno-associated virus to express miR-26a and reduce tumorigenesis in an animal model of hepatocellular carcinoma (76). Issues that still need to be addressed include how to target miR-mimics to the tissue of interest, potential toxicity of AAV vectors, and off-target effects of the miR-mimic.

To treat diseases that are associated with overexpression of specific miRNAs, several approaches have been considered. One of the first strategies, called **antagomirs**, uses chemically modified oligonucleotides that are complementary in sequence to the overexpressed miRNA (77). Antagomirs bind to the miRNA through Watson-Crick base pairing and thereby prevent their association with mRNA targets. To facilitate their entry into cells, antagomirs are modified with cholesterol. **Fig. 16** shows an example of the use of antagomirs in a pre-clinical study of cancer. As discussed earlier, overexpression of the miR-17~92 cluster is frequently

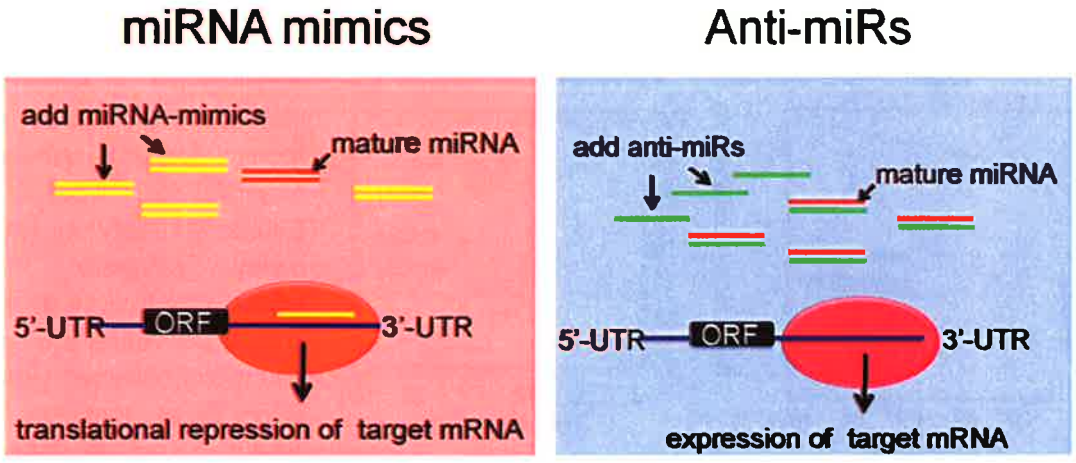


Figure 15. miRNAs as therapeutic targets. Ref: Patel & Noureddine, Curr Opin Nephrol Hyperten, 2012

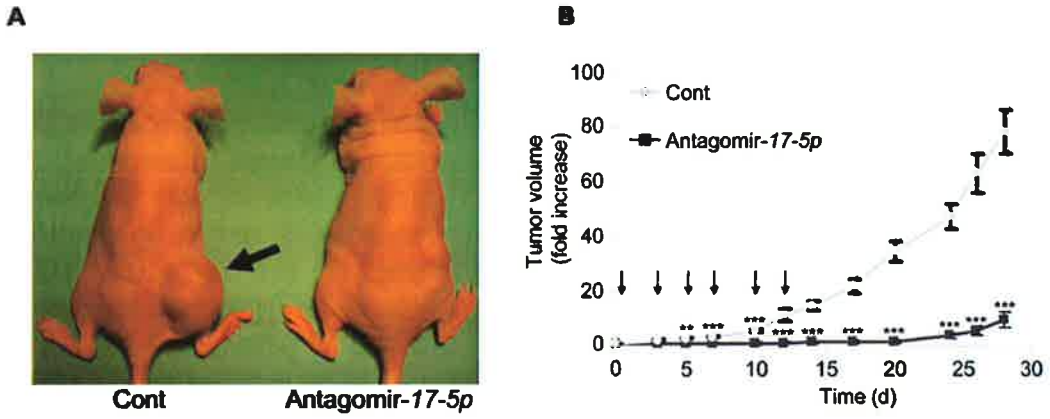


Figure 16. miR-17 antagomir inhibits in vivo tumorigenesis. Ref: Fontana, PLoS One, 2008

observed in cancer. Fontana et al administered a miR-17 antagomir to nude mice that had been implanted with human neuroblastoma cells (42). As shown in the figure, the growth of the neuroblastoma tumor was prevented by the miR-17 antagomir. One encouraging finding in pre-clinical studies using antagomirs is their long half-life and long duration of effect that may persist for weeks after administration is stopped (78). These results raise the hope that miRNA therapeutics will produce durable effects that require infrequent, perhaps monthly, dosing. Another approach to inhibit miRNAs is the use of "**miRNA sponges**" (79). miRNA sponges contain multiple copies of the recognition sequence for a particular miRNA and thereby bind to and sequester the overexpressed miRNA. A third approach is the use of anti-miR oligonucleotides (80). **Anti-miRs** can be shorter, which facilitates their entry into cells. Their chemical backbones contain phosphorothioate oligonucleotides, which makes them resistant to degradation by plasma ribonucleases. In addition, the base pairs are chemically modified using "**locked nucleic acids**" (LNA), which gives them higher affinity despite their shorter length.

First Clinical Trials in Humans

The first clinical trial of a miRNA-based therapeutic in humans has recently been completed. In this study, miR-122 was targeted in patients with chronic hepatitis C virus (HCV) infection. To understand the study, it is first necessary to briefly review the role of miR-122 in the liver. **miR-122** is a liver-specific miRNA that regulates the expression

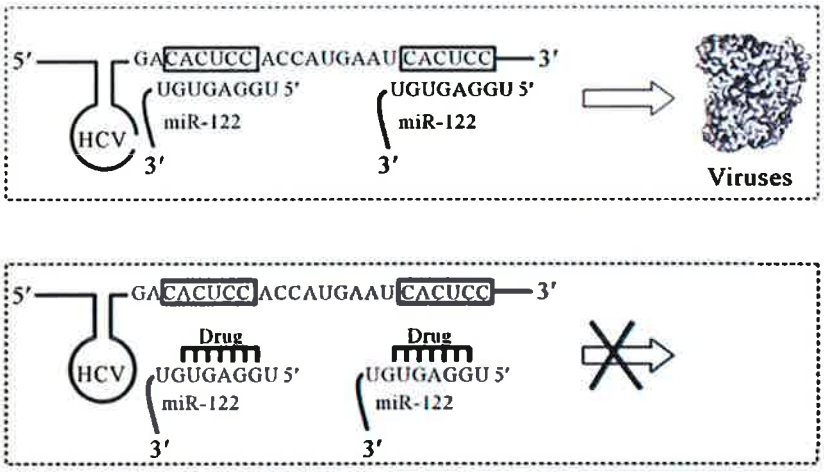


Figure 17. Upper: miR-122 promotes HCV replication. Lower: miR-122 anti-miR inhibits viral replication. Ref: Liu, J Biomed Sci Engineer, 2011

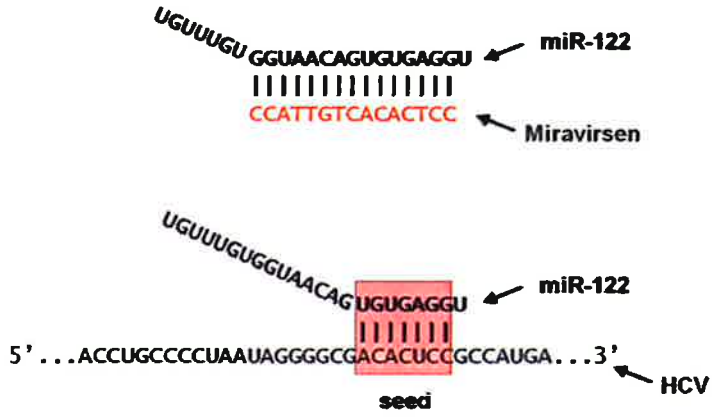


Figure 19. Sequence of miR-122 and anti-miR (miravirsen)

of genes involved in cholesterol metabolism. HCV has subverted the host miRNA machinery to promote viral replication. The HCV RNA genome contains two binding sites for miR-122 located near the 5' end of the genome (**Fig. 17**) (81, 82). In contrast to the usual inhibitory function of miRNAs, binding of miR-122 to the HCV genome increases the accumulation of the virus, possibly by protecting it from the host RNA decay machinery (83). Sequestration of miR-122 with antisense constructs targeting the miRNA reduces the replication of HCV in cultured cells (81). Based on these findings, investigators have developed drugs that target miR-122 for use in patients with chronic HCV infection. The drug that is farthest along in development is called miravirsen (Santaris Pharma). **Miravirsen** (also known as SPC3649) is a 15-mer LNA-modified phosphorothioate oligonucleotide (anti-miR) that is complementary to the 5' end of miR-122. As shown in **Fig. 18**, miravirsen binds to miR-122 and prevents its association with the HCV genome, which inhibits viral replication. Pre-clinical studies in African green monkeys showed that miravirsen is well-tolerated and produces long-lasting decreases in serum cholesterol due to inhibition of enzymes involved in cholesterol biosynthesis (78). Lanford et al at the Southwest National Primate Center near San Antonio, TX tested miravirsen in chimpanzees with chronic HCV infection. They found that miravirsen produced long-lasting suppression of HCV viral load in both the serum and liver (84). Liver histology improved in animals receiving the higher dose of miravirsen. miR-122 is a particularly attractive target for systemic anti-miR therapy since its expression is liver-specific and the effects of inhibition in other tissues should be less of a concern. Indeed, miravirsen was not associated with systemic toxicity in non-human primates (78, 84).

Miravirsen is the **first miRNA-based therapeutic** that has been administered to humans (79). Last month at the European Association for the Study of the Liver (EASL) meeting, Reesink et al reported the final results of a phase 2a clinical trial of miravirsen in patients with chronic HCV infection. The study, sponsored by Santaris Pharma, was a randomized, double-blind placebo-controlled, multicenter study. The eligibility criteria were chronic HCV infection, genotype 1, treatment naïve, and compensated liver disease (no cirrhosis).

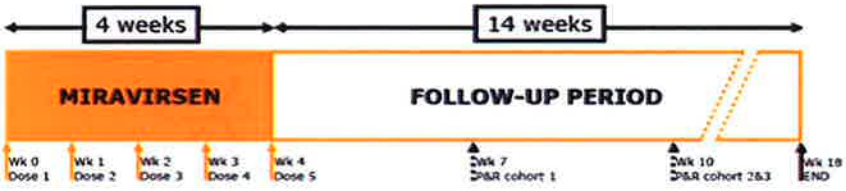
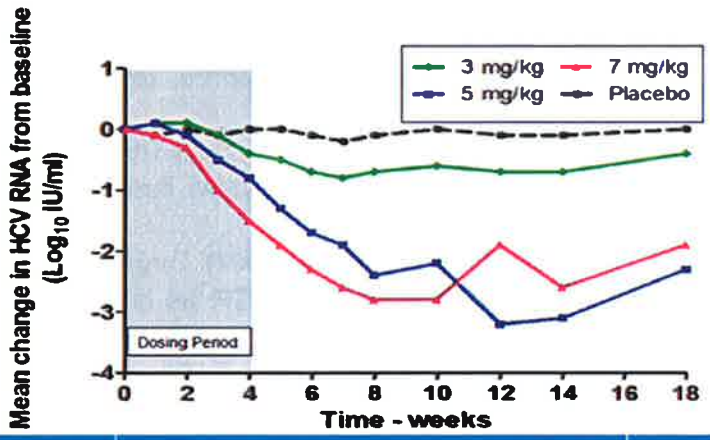


Figure 18. Miravirsen study design

The primary outcomes were safety and tolerability, and the secondary outcomes were viral load, pharmacokinetics/pharmacodynamics, and HCV sequence analysis. Thirty-six subjects were enrolled and randomized to receive five weekly subcutaneous doses of miravirsen (3, 7, or 7 mg/kg) or placebo, then were followed for 14 weeks (**Fig. 19**). Patients were allowed to receive PEGylated interferon/ribavarine 3-6 weeks after the last dose.

Fig. 20 shows that miravirsen produced a dose-dependent decline in HCV viral load. Importantly, the decrease in viral load continued after the administration of miravirsen was stopped, consistent with the ~30-day half-life of the anti-miR. The mean maximum decrease in subjects who received the highest dose was a 3-log reduction. Moreover, five subjects achieved undetectable viral titers. Side-effects were mild, and there were no dose-limiting toxicities. Moreover, deep sequencing of the HCV genome found no evidence of mutations of the miR-122 binding sites that might indicate resistance. These preliminary findings suggest that anti-miRs may be safe and efficacious in humans. Further studies are clearly needed, but miRNA-based therapeutics seem to have a promising future.



Dose Group	Mean Maximum HCV RNA decline IU/mL (SEM) [without SOC]	p value MIR vs placebo
placebo	-0.5 (0.3)	-
3 mg/Kg	-1.2 (0.6)	0.013
5 mg/Kg	-2.9 (1.7)	0.003
7 mg/Kg	-3.0 (1.6)	0.002

Figure 20. Miravirsen decreases HCV viral load

Summary

Since the discovery of *let-7* in 2000, there has been an explosion of interest in the class of small, non-coding RNA molecules known as microRNAs. Studies have revealed that microRNAs comprise the endogenous RNA interference system. Despite their small size, they can bind to target mRNAs and mediate their degradation (David vs. Goliath). MicroRNAs inhibit post-transcriptional gene expression and thereby maintain the abundance of proteins within an optimal range (Goldilocks Principle). Humans express hundreds of different microRNAs, and more than one-third of our genes may be regulated by microRNAs. MicroRNAs play important roles in normal human biology, including embryonic development, stem cells, hematopoiesis, immunity, and homeostasis. Deregulation of microRNAs is observed in many, perhaps all, human diseases, including cancer, tissue fibrosis. MicroRNAs are being explored as possible biomarkers for disease diagnosis and prognosis. Finally, microRNAs are promising therapeutic targets for the treatment of common human diseases.

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