Regulatory RNAs: Future Perspectives in Diagnosis and Personalized Therapy

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Abstract

With potentially up to 1000 microRNAs (miRNAs) present in the human genome, altogether regulating the expression of thousands of genes, one can anticipate that miRNAs will play a significant role in health and disease. Deregulated protein expression induced by a dysfunctional miRNA-based regulatory system is thus expected to lead to the development of serious, if not lethal, genetic diseases. A relationship between miRNAs, Dicer and cancer has recently been suggested. Further investigations will help establish specific causal links between dysfunctional miRNAs and diseases. miRNAs of foreign origin, e.g. viruses, may also be used as specific markers of viral infections. In these cases, miRNA expression profiles could represent a powerful diagnostic tool. Regulatory RNAs may also have therapeutic applications, by which disease-causing genes or viral miRNAs could be neutralized, or functional miRNAs be restored. Will bedside miRNA expression profiling eventually assist physicians in providing patients with accurate diagnosis, personalized therapy and treatment outcome?

1. microRNAs as novel regulators of gene expression

miRNA-guided RNA silencing is a recently discovered gene regulatory process by which endogenous miRNAs mediate translational repression of specific mRNAs through imperfect complementarity. Whereas RNA interference (RNAi) is rather referred to as the process initiated by exogenous small interfering RNAs (siRNAs) that are designed to induce cleavage and degradation of specific mRNAs. siRNAs are thus a powerful tool to knockdown genes potently and specifically (1). Usually chemically synthesized, siRNAs are designed to be perfectly complementary to a targeted mRNA and are formed by two complementary strands of ~21 to 23 nucleotides (nt) with 2-nt 3' overhangs (1). siRNAs are phosphorylated at the 5' termini and hydroxylated at their 3' ends (2), mimicking the endogenous miRNA:miRNA* duplexes resulting from Dicer processing of endogenous miRNA precursors (pre-miRNAs).

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Several chemical modifications can be made to siRNAs to enhance some of their characteristics, such as their stability, cellular uptake or intracellular distribution. Modifications affecting the second carbon of the pentose sugar have been widely characterized, such as 2'-O-Methylation (2'OMe) and deoxynucleotides (3). siRNAs can also be obtained by the cleavage by Dicer of short hairpin RNAs (shRNAs), which are transcribed from a vector or a PCR product containing this particular sequence. shRNAs are structurally homologous to the endogenous miRNAs precursors, with the difference that their stem is usually perfectly complementary. It is important to note that, when introduced into mammalian cells, siRNAs do not seem to induce an interferon response (1), opening up the possibilities for therapeutic applications.

miRNA genes can be found as clusters forming their own transcriptional units (4,5). For miRNAs that coincide with protein-encoding genes, the majority are found within introns (6). These observations strongly suggest that some miRNAs are coordinately expressed in parallel with their host proteins. Primary transcripts (pri-miRNAs) possess the signature of RNA polymerase (pol) II transcribed RNAs, characterized by a 5' methylguanosine cap and a 3' poly(A) tail (7,8). As shown in Figure 1, the first processing step is initiated by the nuclear ribonuclease (RNase) III Drosha and produces a ~60 to 70-nt stem loop named pre-miRNAs (4,9). The Drosha cleavage products harbor a classical 2-nt 3' overhangs and a 5' phosphate that characterize cleavage of dsRNA substrates by members of the RNase III family (10–12). This step is carried out in collaboration with the DiGeorge syndrome critical region 8 (DGCR8) protein; this heterodimeric complex is known as the microprocessor (13–16). The pre-miRNA is then exported from the nucleus to the cytoplasm by the Ran-GTP dependent transporter exportin-5 (exp-5) (17–20). A second RNase III enzyme located in the cytoplasm, Dicer (21–24), together with transactivating response RNA-binding protein (TRBP) (25,26), catalyzes the second processing step by cleaving the pre-miRNA at the base of the loop to generate an imperfect miRNA:miRNA* duplex of ~21 to 23-nt, which will be incorporated into an effector ribonucleoprotein (RNP) complex (27), such as the RNA-induced silencing complex (RISC) (28). Dicer and TRBP have recently been shown to be part of a functional RISC, thereby coupling the initiation and effector steps of RNAi (29).

A difference between siRNAs and miRNAs resides in base pairing. While siRNAs show a perfect complementarity between the nucleotides of both strands, excluding the 2-nt 3' overhangs, miRNAs show a variable number of mismatches that may confer a certain degree of instability (30,31). Based on the thermodynamic stability of the duplex, a strand selection process will determine the identity of the strand to be incorporated into a functional miRNA-containing RNP (miRNP) complex (29,32–34). The miRNA strand with the less stable 5' end pairing is loaded into the miRNP, whereas the miRNA*, also called the passenger strand, is usually destroyed (33).

First identified in *Drosophila* S2 cells (28), the RISC is the best characterized RNP complex in RNA silencing. It has been reported to contain Argonaute 2 (Ago2) (35), the staphylococcal nuclease Tudor (Tudor-SN) (36), the vasa intronic gene (VIG) protein (37) and the fragile X mental retardation protein (FMRP) (37,38). Although the function of these proteins within the RISC remains unclear, Ago2 has been shown to play a central role in RISC activity (39–41). The PAZ domain of Ago2, a domain also present in Dicer, recognizes

and binds the 3' end of single-stranded RNAs (42–44), whereas the PIWI domain binds the 5' end (45). PIWI, which shows structural similarity to RNase H (46), is responsible for the mRNA cleavage activity in the RISC (47). Human Ago2 binding to a mature miRNA can lead to the cleavage or translational repression of the mRNA target (48–50).

2. Potential regulatory roles for miRNAs

As of October 2005, 326 different miRNAs have been identified in human, which corresponds to ~2% of the genome (<http://microrna.sanger.ac.uk/>) (51). Little is known about their biological functions, but they may regulate more than 30% of the genes (52,53). miRNAs bind to a mRNA by an imperfect nucleotide base pairing. Generally, the critical miRNA:mRNA pairing region, referred to as the miRNA seed, is usually located in the 3' nontranslated region (NTR) of specific mRNAs and comprised of miRNA positions 2 to 8 in the 5' to 3' orientation. Perfect pairing of this miRNA region is a key parameter used by computational approaches that aim at predicting potential mRNA targets of miRNAs. Pairing of the miRNA 3' region appears to be less important, but may compensate a weaker binding of the 5' region (54). In humans, mRNA regulation by miRNAs is believed to consist mainly in translational repression, although a recent study reported that miRNAs downregulate a greater number of transcripts than previously appreciated (55). miRNA regulation of mRNAs is complex. For example, a given miRNA can regulate several different mRNAs. Conversely, a specific mRNA can be regulated by more than one coexpressed miRNA. This may allow for a finely tuned expression of a gene product whose levels are critical for normal cell function.

Experimental demonstration of a relevant miRNA:mRNA target interaction is arduous to achieve; only a few combinations have been experimentally validated thus far. A study of the let-7:lin-41 interaction in *C. elegans* by Vella et al. (54) has improved our understanding of the requirements for miRNA binding. The miRNA let-7 possesses 6 putative binding sites in the 3'NTR region of the mRNA lin-41. At least two sites are necessary for downregulation of lin-41 expression. The intervening 27-nt sequence also appears to be important for miRNA regulation (54), suggesting that miRNA:mRNA interactions do not rely solely to the regions of complementarity. A better comprehension of the factors involved in mRNA recognition by miRNAs will help conceive better predictive methods that are useful to limit the number of potential mRNA targets to be investigated.

Prior to initiating studies aimed at characterizing a miRNA:mRNA interaction of interest, computational approaches remain the method of choice to identify mRNAs possibly subjected to miRNA regulation, or to identify miRNAs possibly regulating a mRNA. Several different algorithms have been created and each of them uses different parameters for the sequence requirement of a miRNA:target interaction. They are designed to search for the miRNA seed and to determine the free energy of the interaction. They can also look for phylogenetic conservation and for more than one miRNA binding site in a given 3'NTR. Some bioinformatic predictive tools are available on internet: Miranda (<http://www.microrna.org>) (56), TargetScan (<http://genes.mit.edu/targetscan/>) (57) and Diana MicroT (<http://diana.cslab.ece.ntua.gr/>) (58). A new database web site, Argonaute (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/>) (59), provides to scientists relevant

information on different predictive algorithms, miRNA identification and RNAi pathway components.

3. miRNAs and their potential involvement in diseases

miRNAs have been shown to regulate an increasing number of cellular processes. They can regulate development, cell proliferation, apoptosis and other important physiological processes, as reviewed by Ouellet et al. (60). Given their recognized importance in gene regulation, a link between miRNAs and several major diseases is expected. Defects in miRNA-mediated regulation of mRNA translation may lead to overexpression of specific proteins, which accumulation may cause diseases (see Figure 1). This may be the case for mutated miRNA or miRNA-binding site on the regulated mRNA that can lead to a loss of mRNA translational control.

Clinical situations of genomic instability have brought support for a role of miRNAs in oncogenesis, as human miRNA genes have been found in fragile sites involved in cancer (61). In the highly malignant human brain tumor glioblastoma, a strong overexpression of miR-21 has been observed (62). This miRNA has been found to suppress apoptosis in this tumor, thereby contributing to the tumorigenesis process (62). In chronic lymphocytic leukemias (CLL), the genomic region containing miR-15a and miR16-1 is deleted or downregulated (63). The absence of these regulatory miRNAs allows for the overexpression of the anti-apoptotic Bcl2 protein, which helps evade apoptosis (64). miR-143 and miR-145 are downregulated in various human cancer cell lines, particularly those established from colorectal tumors (65). Potential targets of these miRNAs have been previously implicated in oncogenesis (65).

miR-155, whose precursor (pre-miR-155) was initially found to be highly expressed in pediatric Burkitt Lymphoma (BL) (66), was recently shown to be absent in primary cases of BL (67). Although the exact link between miR-155 and BL is unclear, changes in miR-155 levels may clearly influence expression of its target genes, which remain to be identified.

The miR-17-92 cluster is often overexpressed in tumor samples from B cells lymphomas (68) and human lung cancer cell lines when compared to normal cell lines (69). These studies revealed that the miR-17-92 cluster can act as a potential human oncogene. The targets for this miRNA cluster, as predicted by using TargetScan (57), include tumor suppressor genes, suggesting that miR-17-92 overexpression can downregulate expression of these suppressor genes, and favor tumorigenesis (69).

Members of the let-7 miRNA gene family are deleted in different forms of cancers (61), supporting their implication in oncogenesis. A reduction of let-7 expression has been observed in human lung tumor samples or cancer cell lines (70). In this study, patients showing a reduced let-7 expression had the worst prognosis after a potentially curative resection. In *C. elegans*, let-7 regulates let-60, the orthologue of the human *RAS* oncogene. Bioinformatic analyses revealed that the three human *RAS* genes contain multiple let-7 binding sites, suggesting that let-7 may also regulate *RAS* expression in human. This is supported by an association between let-7 downregulation and an increased expression of

RAS protein (71), further implicating the loss of let-7-regulated RAS expression during the development of lung cancer.

Altered expression of a protein component of the miRNA-guided RNA silencing pathway may have a global impact on the expression of genes regulated by miRNAs. Indeed, a decreased expression of the RNase III Dicer was observed in non small cell lung cancer (NSCLC) obtained from patients. This reduction is also associated with shorter postoperative survival (72).

The emerging causal link between miRNAs and diseases suggest that health may lie on a delicate balance between the expression of miRNAs and that of the genes they are regulating. A shift in this balance may lead to a pathogenic downregulation or overexpression of the mRNA-encoded protein. Diseased organs or tissues may exhibit a unique set of miRNA expression profile, which could be used in improving diagnosis of diseases (see Figure 2), such as cancer (73,74).

In addition to cancer, cellular miRNAs can be implicated in host/virus interactions. miR-32 has been shown to restrict accumulation of the retrovirus primate foamy virus type 1 (PFV-1) in human cells (75). This virus encodes Tas, a protein inhibiting RNA silencing in mammalian cells, probably to attenuate the suppressive effects of miR-32.

As for miR-122, which is highly and specifically expressed in the liver, its sequestration caused a marked loss of autonomous replicating hepatitis C viral (HCV) RNAs (76). This miRNA was found to facilitate replication of HCV by targeting the 5'NTR of its genome (76).

Bioinformatical analyses aimed at identifying human immunodeficiency virus type 1 (HIV-1) genes regulated by human miRNA targets yielded five potential targets: miR-29a and miR-29b may target the *nef* gene, miR-149, the *vpr* gene, miR-378, the *vpu* gene and miR-324-5p, the *vif* gene. Expression of these miRNAs was verified by microarray profiling of human T cells, hosts of HIV-1 infection (77).

4. miRNAs as potential therapeutic targets

The possible pathogenic consequences of a miRNA deregulation, associated with either miRNA downregulation or overexpression, makes them potential therapeutic targets. For example, in cases of BL cancer, miR-155 is highly overexpressed (66,67). In this case, its neutralization by an antisense strategy using 2'OMe oligoribonucleotides perfectly complementary to the miR-155 sequence could be envisioned (78).

It is known that viruses like Simian virus 40 (SV40) (79), Epstein-Barr virus (EBV) (80) and Kaposi's sarcoma (KS)-associated herpesvirus (KSHV) (81) encode viral miRNAs. These small RNAs may directly influence specific host and/or viral gene expression, possibly affecting antiviral host defenses or replication of the virus. Thus, antisense 2'OMe oligoribonucleotides may also find applications as anti-viral therapeutic agents.

In contrast, diseases, such as cancer, may also arise from a downregulated miRNA expression, as discussed for that of let-7 in the previous section. In that case, the objective would be the restoration of miRNA expression in the affected organ or tissue. Among the currently available approaches for such an intervention are the administration of miRNA duplexes, shRNA encoding cassettes or even a viral vector encoding an shRNA.

Small regulatory RNAs may also be used as therapeutic agents in treating non-miRNA mediated pathologies caused by overexpression of a specific gene. Here, approaches based on the administration of gene-specific siRNAs may be indicated.

Several obstacles that need to be surmounted before the launch of an RNAi-based therapy to fight genetic diseases are currently being addressed. For instance, the cost of an shRNA or siRNA therapy would be relatively high considering its relative instability, the amount required to achieve therapeutic levels in a 75-kg human being and the anticipated duration and frequency of the treatment. As for the use of viral vectors for stable expression of therapeutic RNAs *in vivo*, it may face uncertainties related to gene therapy, such as immunological issues and long-term undesirable effects.

In vivo delivery of therapeutic RNAs represents one of the key hurdles to the use of RNAi as a therapeutic approach. Several aspects of delivery, such as targeting of specific tissues, cellular uptake and genomic integration, in addition to the issues of specificity, need to be either optimized or developed. Among the delivery methods being developed are cationic carriers, electroporation and lentiviral-based approaches, as reviewed by Lu et al. (82). From the intense research activities on the fundamental and therapeutic aspects of RNAi in the academia as well as in biotechnology firms, we can anticipate major and significant advances in the field of therapeutic RNAs in a near future.

5. Personalized medicine based on monitoring miRNA expression

Several studies have shown that some diseases, such as cancer, are associated with a change in miRNA expression levels (63,70). But, whether these changes are the cause or the effect of cancer remains to be determined. As for viral infections, miRNAs expressed from viruses like EBV (80) and KSHV (81) may influence viral and host gene expression. These observations suggest that monitoring of miRNA expression levels in an affected organ or tissue may be used for diagnostic of a disease or an infection.

However, a clear link between an altered miRNA expression and a given disease first need to be established. Comparative analyses require a “normal” range of miRNA expression in healthy organs or tissues to be defined. A number of methods that allow detection and quantification of the small miRNAs have emerged. A method to be useful for diagnostic purposes would need to be fast, reliable, reproducible, very sensitive (to detect miRNAs of lower abundance) and very specific, in order to discriminate between paralogous miRNAs that sometimes differ only from one nt.

Northern blotting is a standard procedure to detect miRNAs. However, this method may not be sensitive enough, as some miRNAs are undetectable by Northern blot. In addition, it requires relatively large amounts of RNA, several separation and hybridization steps, and

isotopic detection, which are far from ideal for clinical diagnostic. It is possible to improve the efficiency of Northern blot analyses by using locked nucleic acid (LNA)-modified oligonucleotide probes (83), which improve sensitivity by 10-fold via enhanced hybridization properties, and by using digoxigenin-labeled RNA probes to allow rapid (minutes to hours) and nonisotopic detection of miRNAs (84). LNA-modified oligonucleotides have also been used successfully to probe the presence of miRNAs in animal embryos *in situ* (85), an approach that could be applicable to cancer diagnosis.

In 2004, Hartig et al. published a method using signal-amplifying ribozymes to detect miRNAs (86). This method is sequence-specific and highly sensitive, with a detection limit of 50 fmol miRNA in the reaction mixture. The probes that were used had been designed for detection of nucleic acids *in vivo*. However, their stability must be improved to avoid ribonuclease-mediated degradation.

Another method to detect and quantify miRNAs is the primer-extension PCR assay (87). In this assay, the first primer is used to convert an RNA template into cDNA in order to introduce a universal PCR binding site and to extend the cDNA to facilitate subsequent monitoring by quantitative PCR (qPCR). The reverse primer is LNA-modified to increase hybridization affinity and improve amplification. This method is inexpensive, sensitive (in the femtomolar range) and allows discrimination between miRNA family members. Recently, a method to quantify miRNA gene expression with a single molecule, called the Direct miRNA assay, has been published (88). It uses two LNA-DNA oligonucleotide probes hybridized to the miRNA of interest. Since these oligos are spectrally distinguishable, every single tagged molecule can be directly counted on a detection instrument. This assay is fast, sensitive and specific.

All the methods described above cannot be used to study the overall expression profile of miRNAs. For that purpose, a modified Invader assay can be used (89). This method is based on the hybridization of a miRNA to two desoxyoligonucleotides (probe and invasive oligonucleotide) that generates a structure that is specifically cleaved by a 5' nuclease (Cleavase). The released invasive oligonucleotide serves in a second cleavage reaction, which involves a fluorescent-labeled oligonucleotide substrate linked to a dye quencher. Thus, the level of miRNA can be measured by a simple fluorometric assay. This assay can be performed in unfractionated detergent lysates, is fast (2–3 hours incubation time) and allows sensitive and specific high-throughput screening analyses.

Finally, the sensitive and semi-quantitative microarrays are also suitable for high throughput detection of miRNAs. Several studies using this technique have been reported during the last few years, with some of them adapting the assay to make it more specific and sensitive (90–92).

As illustrated in Figure 2, we can imagine that, in a near future, monitoring of miRNA expression will be used as a routine test for diagnosis and treatment of diseases. The first step would be the sampling of the affected organ or tissue, either by biopsy or withdrawal of body fluids such as plasma, saliva or semen. The next steps would be RNA extraction, if necessary, followed by monitoring and analysis of the results by comparison with a

normalized miRNA expression profile, as deduced from data obtained from a bank of normal healthy tissues or fluids. Interpretation of the results would be useful to the physician for establishing a diagnosis and offering various therapeutic avenues to the patient. If the disease is caused by a virus, the treatment would possibly aim at neutralizing the function of viral miRNAs. Whereas if the disease is caused by an abnormal miRNA or gene expression, the treatment would consist either at restoring miRNA expression or targeting the mRNA of the disease-causing gene for degradation.

From all the evidences gathered thus far, we have all the reasons to be enthusiastic about the eventual use of regulatory RNAs in diagnosis and personalized therapy.

Acknowledgments

We are grateful to Gilles Chabot for graphic design. M.P.P. and L.-A.G. are supported by doctoral and master studentships from Natural Sciences and Engineering Research Council of Canada (NSERC), respectively. P.P. is a New Investigator of the Canadian Institutes of Health Research (CIHR) and The Arthritis Society. This work was financially supported by a Discovery grant from NSERC (262938-03) and a grant from Health Canada/CIHR (EOP-64706).

References

1. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*. 2001; 411:494–498. [PubMed: 11373684]
2. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev*. 2001; 15:188–200. [PubMed: 11157775]
3. Chiu YL, Rana TM. siRNA function in RNAi: a chemical modification analysis. *Rna*. 2003; 9:1034–1048. [PubMed: 12923253]
4. Lee Y, Jeon K, Lee JT, Kim S, Kim VN. MicroRNA maturation: stepwise processing and subcellular localization. *Embo J*. 2002; 21:4663–4670. [PubMed: 12198168]
5. Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A. Identification of mammalian microRNA host genes and transcription units. *Genome Res*. 2004; 14:1902–1910. [PubMed: 15364901]
6. Ying SY, Lin SL. Intronic microRNAs. *Biochem Biophys Res Commun*. 2005; 326:515–520. [PubMed: 15596130]
7. Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, Kim VN. MicroRNA genes are transcribed by RNA polymerase II. *Embo J*. 2004; 23:4051–4060. [PubMed: 15372072]
8. Cai X, Hagedorn CH, Cullen BR. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *Rna*. 2004; 10:1957–1966. [PubMed: 15525708]
9. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, et al. The nuclear RNase III Drosha initiates microRNA processing. *Nature*. 2003; 425:415–419. [PubMed: 14508493]
10. Basyuk E, Suavet F, Doglio A, Bordonne R, Bertrand E. Human let-7 stem-loop precursors harbor features of RNase III cleavage products. *Nucleic Acids Res*. 2003; 31:6593–6597. [PubMed: 14602919]
11. Zeng Y, Yi R, Cullen BR. Recognition and cleavage of primary microRNA precursors by the nuclear processing enzyme Drosha. *Embo J*. 2005; 24:138–148. [PubMed: 15565168]
12. Filippov V, Soloviyev V, Filippova M, Gill SS. A novel type of RNase III family proteins in eukaryotes. *Gene*. 2000; 245:213–221. [PubMed: 10713462]
13. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ. Processing of primary microRNAs by the Microprocessor complex. *Nature*. 2004; 432:231–235. [PubMed: 15531879]
14. Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, Shiekhattar R. The Microprocessor complex mediates the genesis of microRNAs. *Nature*. 2004; 432:235–240. [PubMed: 15531877]

15. Han J, Lee Y, Yeom KH, Kim YK, Jin H, Kim VN. The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev.* 2004; 18:3016–3027. [PubMed: 15574589]
16. Landthaler M, Yalcin A, Tuschl T. The human DiGeorge syndrome critical region gene 8 and Its D. melanogaster homolog are required for miRNA biogenesis. *Curr Biol.* 2004; 14:2162–2167. [PubMed: 15589161]
17. Bohnsack MT, Czaplinski K, Gorlich D. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *Rna.* 2004; 10:185–191. [PubMed: 14730017]
18. Brownawell AM, Macara IG. Exportin-5, a novel karyopherin, mediates nuclear export of double-stranded RNA binding proteins. *J Cell Biol.* 2002; 156:53–64. [PubMed: 11777942]
19. Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U. Nuclear export of microRNA precursors. *Science.* 2004; 303:95–98. [PubMed: 14631048]
20. Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev.* 2003; 17:3011–3016. [PubMed: 14681208]
21. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature.* 2001; 409:363–366. [PubMed: 11201747]
22. Provost P, Dishart D, Doucet J, Frendewey D, Samuelsson B, Radmark O. Ribonuclease activity and RNA binding of recombinant human Dicer. *Embo J.* 2002; 21:5864–5874. [PubMed: 12411504]
23. Zhang H, Kolb FA, Brondani V, Billy E, Filipowicz W. Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. *Embo J.* 2002; 21:5875–5885. [PubMed: 12411505]
24. Zhang H, Kolb FA, Jaskiewicz L, Westhof E, Filipowicz W. Single processing center models for human Dicer and bacterial RNase III. *Cell.* 2004; 118:57–68. [PubMed: 15242644]
25. Haase AD, Jaskiewicz L, Zhang H, Laine S, Sack R, Gatignol A, Filipowicz W. TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. *EMBO Rep.* 2005; 6:961–967. [PubMed: 16142218]
26. Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K, Shiekhattar R. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature.* 2005; 436:740–744. [PubMed: 15973356]
27. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* 2004; 116:281–297. [PubMed: 14744438]
28. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature.* 2000; 404:293–296. [PubMed: 10749213]
29. Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R. Human RISC Couples MicroRNA Biogenesis and Posttranscriptional Gene Silencing. *Cell.* 2005; 123:631–640. [PubMed: 16271387]
30. Khvorova A, Reynolds A, Jayasena SD. Functional siRNAs and miRNAs exhibit strand bias. *Cell.* 2003; 115:209–216. [PubMed: 14567918]
31. Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD. Asymmetry in the assembly of the RNAi enzyme complex. *Cell.* 2003; 115:199–208. [PubMed: 14567917]
32. Liu Q, Rand TA, Kalidas S, Du F, Kim HE, Smith DP, Wang X. R2D2, a bridge between the initiation and effector steps of the *Drosophila* RNAi pathway. *Science.* 2003; 301:1921–1925. [PubMed: 14512631]
33. Matranga C, Tomari Y, Shin C, Bartel DP, Zamore PD. Passenger-Strand Cleavage Facilitates Assembly of siRNA into Ago2-Containing RNAi Enzyme Complexes. *Cell.* 2005; 123:607–620. [PubMed: 16271386]
34. Mourelatos Z, Dostie J, Paushkin S, Sharma A, Charroux B, Abel L, Rappsilber J, Mann M, Dreyfuss G. miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. *Genes Dev.* 2002; 16:720–728. [PubMed: 11914277]
35. Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ. Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science.* 2001; 293:1146–1150. [PubMed: 11498593]

36. Caudy AA, Ketting RF, Hammond SM, Denli AM, Bathoorn AM, Tops BB, Silva JM, Myers MM, Hannon GJ, Plasterk RH. A micrococcal nuclease homologue in RNAi effector complexes. *Nature*. 2003; 425:411–414. [PubMed: 14508492]
37. Caudy AA, Myers M, Hannon GJ, Hammond SM. Fragile X-related protein and VIG associate with the RNA interference machinery. *Genes Dev*. 2002; 16:2491–2496. [PubMed: 12368260]
38. Ishizuka A, Siomi MC, Siomi H. A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. *Genes Dev*. 2002; 16:2497–2508. [PubMed: 12368261]
39. Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song JJ, Hammond SM, Joshua-Tor L, Hannon GJ. Argonaute2 is the catalytic engine of mammalian RNAi. *Science*. 2004; 305:1437–1441. [PubMed: 15284456]
40. Rand TA, Petersen S, Du F, Wang X. Argonaute2 Cleaves the Anti-Guide Strand of siRNA during RISC Activation. *Cell*. 2005; 123:621–629. [PubMed: 16271385]
41. Rivas FV, Tolia NH, Song JJ, Aragon JP, Liu J, Hannon GJ, Joshua-Tor L. Purified Argonaute2 and an siRNA form recombinant human RISC. *Nat Struct Mol Biol*. 2005; 12:340–349. [PubMed: 15800637]
42. Ma JB, Ye K, Patel DJ. Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. *Nature*. 2004; 429:318–322. [PubMed: 15152257]
43. Song JJ, Liu J, Tolia NH, Schneiderman J, Smith SK, Martienssen RA, Hannon GJ, Joshua-Tor L. The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. *Nat Struct Biol*. 2003; 10:1026–1032. [PubMed: 14625589]
44. Yan KS, Yan S, Farooq A, Han A, Zeng L, Zhou MM. Structure and conserved RNA binding of the PAZ domain. *Nature*. 2003; 426:468–474. [PubMed: 14615802]
45. Ma JB, Yuan YR, Meister G, Pei Y, Tuschl T, Patel DJ. Structural basis for 5'-end-specific recognition of guide RNA by the A. fulgidus Piwi protein. *Nature*. 2005; 434:666–670. [PubMed: 15800629]
46. Parker JS, Roe SM, Barford D. Crystal structure of a PIWI protein suggests mechanisms for siRNA recognition and slicer activity. *Embo J*. 2004; 23:4727–4737. [PubMed: 15565169]
47. Song JJ, Smith SK, Hannon GJ, Joshua-Tor L. Crystal structure of Argonaute and its implications for RISC slicer activity. *Science*. 2004; 305:1434–1437. [PubMed: 15284453]
48. Pillai RS, Artus CG, Filipowicz W. Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. *Rna*. 2004; 10:1518–1525. [PubMed: 15337849]
49. Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, Basyuk E, Bertrand E, Filipowicz W. Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science*. 2005; 309:1573–1576. [PubMed: 16081698]
50. Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell*. 2004; 15:185–197. [PubMed: 15260970]
51. Griffiths-Jones S. The microRNA Registry. *Nucleic Acids Res*. 2004; 32:D109–111. [PubMed: 14681370]
52. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*. 2005; 120:15–20. [PubMed: 15652477]
53. Xie X, Lu J, Kulbokas EJ, Golub TR, Mootha V, Lindblad-Toh K, Lander ES, Kellis M. Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals. *Nature*. 2005; 434:338–345. [PubMed: 15735639]
54. Vella MC, Reinert K, Slack FJ. Architecture of a validated microRNA: target interaction. *Chem Biol*. 2004; 11:1619–1623. [PubMed: 15610845]
55. Lim LP, Lau NC, Garrett-Engle P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*. 2005; 433:769–773. [PubMed: 15685193]
56. John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human MicroRNA targets. *PLoS Biol*. 2004; 2:e363. [PubMed: 15502875]
57. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. *Cell*. 2003; 115:787–798. [PubMed: 14697198]

58. Kiriakidou M, Nelson PT, Kouranov A, Fitziev P, Bouyioukos C, Mourelatos Z, Hatzigeorgiou A. A combined computational-experimental approach predicts human microRNA targets. *Genes Dev.* 2004; 18:1165–1178. [PubMed: 15131085]
59. Shahi P, Loukianiouk S, Bohne-Lang A, Kenzelmann M, Kuffer S, Maertens S, Eils R, Grone HJ, Gretz N, Brors B. Argonaute--a database for gene regulation by mammalian microRNAs. *Nucleic Acids Res.* 2006; 34:D115–118. [PubMed: 16381827]
60. Ouellet DL, Perron MP, Plante P, Provost P. MicroRNAs in gene regulation: when the smallest governs it all. *J Biomed Biotech.* 2006 (Submitted).
61. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci U S A.* 2004; 101:2999–3004. [PubMed: 14973191]
62. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res.* 2005; 65:6029–6033. [PubMed: 16024602]
63. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A.* 2002; 99:15524–15529. [PubMed: 12434020]
64. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A.* 2005; 102:13944–13949. [PubMed: 16166262]
65. Michael MZ, SMOC, van Holst Pellekaan NG, Young GP, James RJ. Reduced accumulation of specific microRNAs in colorectal neoplasia. *Mol Cancer Res.* 2003; 1:882–891. [PubMed: 14573789]
66. Metzler M, Wilda M, Busch K, Viehmann S, Borkhardt A. High expression of precursor microRNA-155/BIC RNA in children with Burkitt lymphoma. *Genes Chromosomes Cancer.* 2004; 39:167–169. [PubMed: 14695998]
67. Kluiver J, Haralambieva E, de Jong D, Blokzijl T, Jacobs S, Kroesen BJ, Poppema S, van den Berg A. Lack of BIC and microRNA miR-155 expression in primary cases of Burkitt lymphoma. *Genes Chromosomes Cancer.* 2006; 45:147–153. [PubMed: 16235244]
68. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ, et al. A microRNA polycistron as a potential human oncogene. *Nature.* 2005; 435:828–833. [PubMed: 15944707]
69. Hayashita Y, Osada H, Tatematsu Y, Yamada H, Yanagisawa K, Tomida S, Yatabe Y, Kawahara K, Sekido Y, Takahashi T. A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. *Cancer Res.* 2005; 65:9628–9632. [PubMed: 16266980]
70. Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res.* 2004; 64:3753–3756. [PubMed: 15172979]
71. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ. RAS is regulated by the let-7 microRNA family. *Cell.* 2005; 120:635–647. [PubMed: 15766527]
72. Karube Y, Tanaka H, Osada H, Tomida S, Tatematsu Y, Yanagisawa K, Yatabe Y, Takamizawa J, Miyoshi S, Mitsudomi T, et al. Reduced expression of Dicer associated with poor prognosis in lung cancer patients. *Cancer Sci.* 2005; 96:111–115. [PubMed: 15723655]
73. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, et al. MicroRNA expression profiles classify human cancers. *Nature.* 2005; 435:834–838. [PubMed: 15944708]
74. Jiang J, Lee EJ, Gusev Y, Schmittgen TD. Real-time expression profiling of microRNA precursors in human cancer cell lines. *Nucleic Acids Res.* 2005; 33:5394–5403. [PubMed: 16192569]

75. Lecellier CH, Dunoyer P, Arar K, Lehmann-Che J, Eyquem S, Himber C, Saib A, Voinnet O. A cellular microRNA mediates antiviral defense in human cells. *Science*. 2005; 308:557–560. [PubMed: 15845854]
76. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science*. 2005; 309:1577–1581. [PubMed: 16141076]
77. Hariharan M, Scaria V, Pillai B, Brahmachari SK. Targets for human encoded microRNAs in HIV genes. *Biochem Biophys Res Commun*. 2005; 337:1214–1218. [PubMed: 16236258]
78. Cheng AM, Byrom MW, Shelton J, Ford LP. Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic Acids Res*. 2005; 33:1290–1297. [PubMed: 15741182]
79. Sullivan CS, Grundhoff AT, Tevethia S, Pipas JM, Ganem D. SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells. *Nature*. 2005; 435:682–686. [PubMed: 15931223]
80. Pfeffer S, Zavolan M, Grasser FA, Chien M, Russo JJ, Ju J, John B, Enright AJ, Marks D, Sander C, et al. Identification of virus-encoded microRNAs. *Science*. 2004; 304:734–736. [PubMed: 15118162]
81. Samols MA, Hu J, Skalsky RL, Renne R. Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. *J Virol*. 2005; 79:9301–9305. [PubMed: 15994824]
82. Lu PY, Xie F, Woodle MC. In vivo application of RNA interference: from functional genomics to therapeutics. *Adv Genet*. 2005; 54:117–142. [PubMed: 16096010]
83. Valoczi A, Hornyik C, Varga N, Burgyan J, Kauppinen S, Havelda Z. Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes. *Nucleic Acids Res*. 2004; 32:e175. [PubMed: 15598818]
84. Ramkissoon SH, Mainwaring LA, Sloand EM, Young NS, Kajigaya S. Nonisotopic detection of microRNA using digoxigenin labeled RNA probes. *Mol Cell Probes*. 2005 [Epub ahead of print].
85. Kloosterman WP, Wienholds E, de Bruijn E, Kauppinen S, Plasterk RH. In situ detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes. *Nat Methods*. 2006; 3:27–29. [PubMed: 16369549]
86. Hartig JS, Grune I, Najafi-Shoushtari SH, Famulok M. Sequence-specific detection of MicroRNAs by signal-amplifying ribozymes. *J Am Chem Soc*. 2004; 126:722–723. [PubMed: 14733539]
87. Raymond CK, Roberts BS, Garrett-Engle P, Lim LP, Johnson JM. Simple, quantitative primer-extension PCR assay for direct monitoring of microRNAs and short-interfering RNAs. *Rna*. 2005; 11:1737–1744. [PubMed: 16244135]
88. Neely LA, Patel S, Garver J, Gallo M, Hackett M, McLaughlin S, Nadel M, Harris J, Gullans S, Rooke J. A single-molecule method for the quantitation of microRNA gene expression. *Nat Methods*. 2006; 3:41–46. [PubMed: 16369552]
89. Allawi HT, Dahlberg JE, Olson S, Lund E, Olson M, Ma WP, Takova T, Neri BP, Lyamichev VI. Quantitation of microRNAs using a modified Invader assay. *Rna*. 2004; 10:1153–1161. [PubMed: 15208450]
90. Nelson PT, Baldwin DA, Searce LM, Oberholtzer JC, Tobias JW, Mourelatos Z. Microarray-based, high-throughput gene expression profiling of microRNAs. *Nat Methods*. 2004; 1:155–161. [PubMed: 15782179]
91. Liang RQ, Li W, Li Y, Tan CY, Li JX, Jin YX, Ruan KC. An oligonucleotide microarray for microRNA expression analysis based on labeling RNA with quantum dot and nanogold probe. *Nucleic Acids Res*. 2005; 33:e17. [PubMed: 15684409]
92. Babak T, Zhang W, Morris Q, Blencowe BJ, Hughes TR. Probing microRNAs with microarrays: tissue specificity and functional inference. *Rna*. 2004; 10:1813–1819. [PubMed: 15496526]

Figure 1.

Importance of miRNA-regulated gene expression in humans. 1) For more than 30% of the genes, miRNAs control the expression of their encoded proteins through recognition of their corresponding mRNAs. 2) miRNA overexpression may accentuate the degree of translation inhibition mediated by miRNAs. 3) Whereas loss of miRNA control, induced by a dysfunctional miRNA or miRNA-binding site, may lead to the overexpression of a specific protein and provoke the development of a disease. 4) miRNAs or siRNAs can also originate from viruses and modulate host gene expression, which may result in facilitation of viral replication. Dysfunction of a protein involved in miRNA biosynthesis or function may affect miRNA expression globally and have more serious, if not lethal, consequences.

Figure 2.
Schematic illustration of the envisioned use of miRNA expression monitoring in personalizing diagnosis and medical treatment.