

The Role of microRNAs in Development and Disease and the Potential for Therapeutic Intervention

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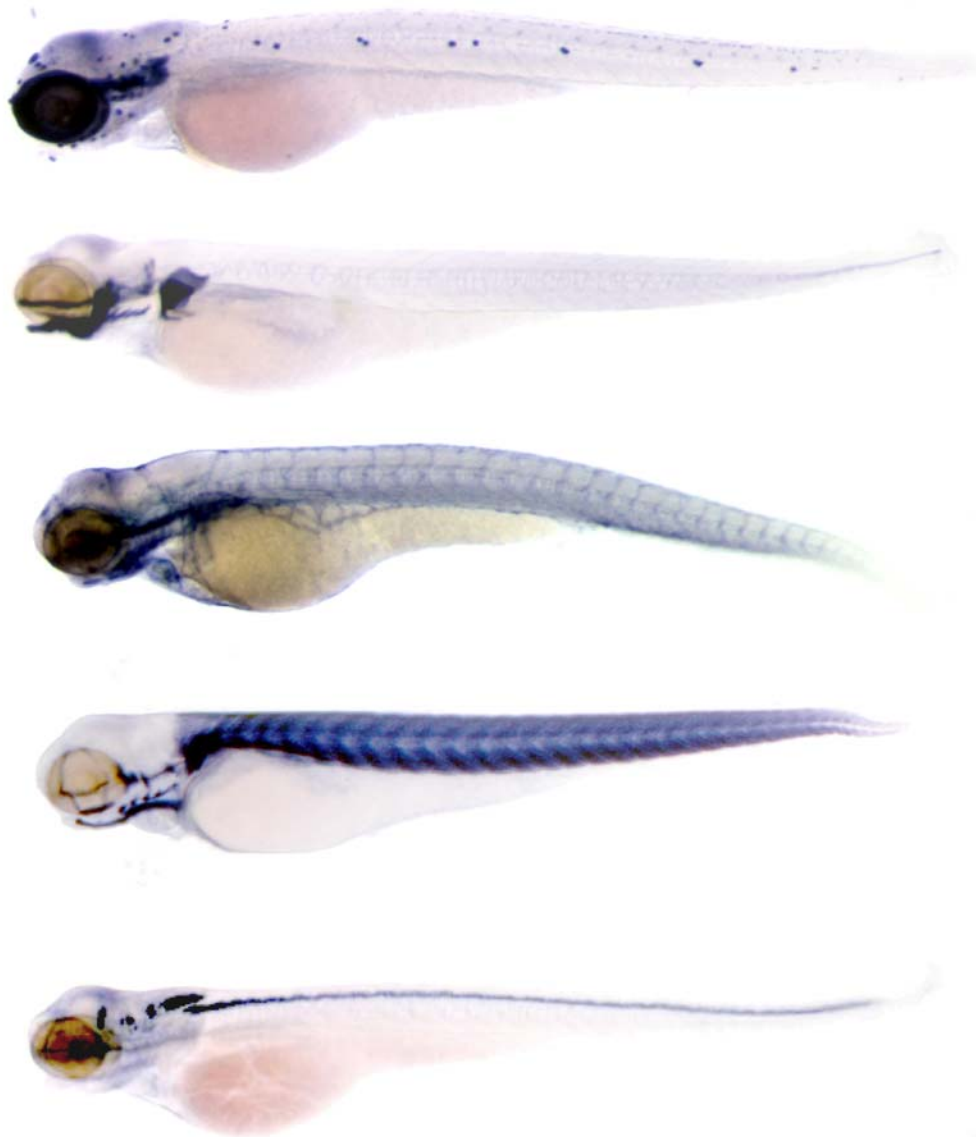


Fig. 1. MicroRNA expression patterns in 3-day-old zebrafish embryos. From Wienholds *et al.* 2005. Science 309: 310-311.

The Central Dogma versus RNA

The expanding inventory of international sequence databases and the concomitant sequencing of more than 350 genomes representing all three domains of life – bacteria, archaea and eukaryota - have been the primary drivers in the process of deconstructing living organisms into comprehensive molecular catalogs of genes, transcripts and proteins. The importance of the genetic variation within a single species has become apparent, extending beyond the completion of genetic blueprints of several important genomes, culminating in the publication of the working draft of the human genome sequence in 2001 (Lander et al. 2001, Venter et al., 2001, Sachidanandam et al. 2001).

On the other hand, the increasing number of detailed, large-scale molecular analyses of transcription originating from the human and mouse genomes along with the recent identification of several types of non-protein-coding RNAs, such as small nucleolar RNAs, siRNAs, microRNAs and antisense RNAs, indicate that the transcriptomes of higher eukaryotes are much more complex than originally anticipated (Wong et al. 2001, *Genome Research* 11: 1975-1977; Kampa et al. 2004, *Genome Research* 14: 331-342).

As a result of the Central Dogma: “DNA makes RNA, and RNA makes protein”, RNAs have for decades been considered as simple molecules that just translate the genetic information into protein. Recently, it has been estimated that although most of the genome is transcribed, almost 97% of the genome does not encode proteins in higher eukaryotes, but putative, non-coding RNAs (Wong et al. 2001, *Genome Research* 11: 1975-1977). The non-coding RNAs (ncRNAs) appear to be particularly well suited for regulatory roles that require highly specific nucleic acid recognition. Therefore, the view of RNA is rapidly changing from the merely informational molecule to comprise a wide variety of structural, informational and catalytic molecules in the cell (Fig. 1).

MicroRNAs – novel regulators of gene expression

MicroRNAs (miRNAs) are an abundant class of short endogenous RNAs that act as post-transcriptional regulators of gene expression by base-pairing with their target mRNAs. The ~ 22 nucleotide (nt) mature miRNAs are processed sequentially from longer hairpin transcripts (Fig. 2) by the RNase III ribonucleases Droscha (Lee et al. 2003) and Dicer (Hutvagner et al. 2001, Ketting et al. 2001). To date more than 3500 microRNAs have been annotated in vertebrates, invertebrates and plants according to the miRBase microRNA database release 8.0 in February 2006 (Griffith-Jones 2004, Griffith-Jones et al. 2006), and many miRNAs that correspond to putative genes have also been identified. More than half of all known mammalian miRNAs are hosted within the introns of pre-mRNAs or long ncRNA transcripts (Rodriguez et al. 2004). Many miRNA genes are arranged in genomic clusters (Lagos-Quintana et al. 2001). For example, ca. 40 % of human miRNA genes appear in clusters of two or more, with the largest cluster of 40 miRNA genes being located in the human imprinted 14q32 domain (Setiz et al. 2004; Altuvia et al. 2005). In plants, 117 miRNA genes have been identified in *Arabidopsis thaliana* while number of miRNAs identified in rice is currently 178 (Griffith-Jones 2004, Griffith-Jones et al. 2006). The identified miRNAs to date represent most likely the tip of the iceberg, and the number of miRNAs might turn out to be very large. Recent bioinformatic predictions combined with array analyses, small RNA cloning and Northern blot validation indicate that the total number of miRNAs in vertebrate genomes is significantly

higher than previously estimated and maybe as many as 1000 (Bentwich et al. 2005, Berezikov et al. 2005, Xie et al. 2005).

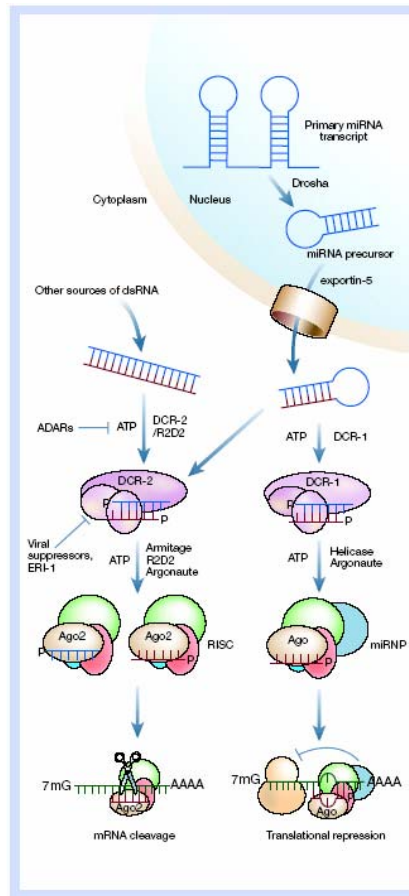


Fig. 2. Model for small RNA-guided post-transcriptional regulation of gene expression. From Meister and Tuschl 2004. Nature 431: 343-349.

The first miRNAs genes to be discovered, *lin-4* and *let-7*, base-pair incompletely to repeated elements in the 3' untranslated regions (UTRs) of heterochronic genes, and control developmental timing in *C. elegans* by regulating translation directly and negatively via antisense RNA-RNA interaction (Lee et al. 1993, Reinhart et al. 2000). The majority of plant miRNAs have perfect or near-perfect complementarity with their target sites and direct RISC-mediated target mRNA cleavage (for review, see Bartel 2004, Fig. 3). A large fraction of the plant miRNAs appears to regulate genes with roles in developmental processes, such as control of meristem identity, cell proliferation, developmental timing and patterning (Kidner and Martienssen 2005). In contrast, most animal miRNAs recognize their target sites located in 3'-UTRs by incomplete base-pairing (Fig. 3), resulting in translational repression of the target genes (Bartel 2004). An increasing body of research shows that animal miRNAs play fundamental biological roles in cell growth and apoptosis (Brennecke et al. 2003), hematopoietic lineage differentiation (Chen et al. 2004), life-span regulation (Boehm and Slack 2005), photoreceptor differentiation (Li and Carthew 2005), homeobox gene regulation (Yekta et al. 2004, Hornstein et al. 2005), neuronal asymmetry (Johnston et al. 2004), insulin secretion (Poy et al. 2004), brain morphogenesis (Giraldez et al. 2005), muscle proliferation and differentiation (Chen, Mandel et al. 2005, Kwon et al. 2005, Sokol and Ambros 2005), cardiogenesis (Zhao et al. 2005) and late embryonic development in vertebrates (Wienholds et al. 2005).

Several studies have identified subclasses of miRNAs directly implicated in the regulation of mammalian brain development and neuronal differentiation (Krichevsky et al. 2003, Miska et al. 2004, Sempere et al. 2004, Smirnova et al. 2005). Interestingly, many neural miRNAs appear to be temporally regulated in cortical cultures copurifying with polyribosomes, suggesting that they may control localized translation of dendrite-specific mRNAs (Kim et al. 2004).

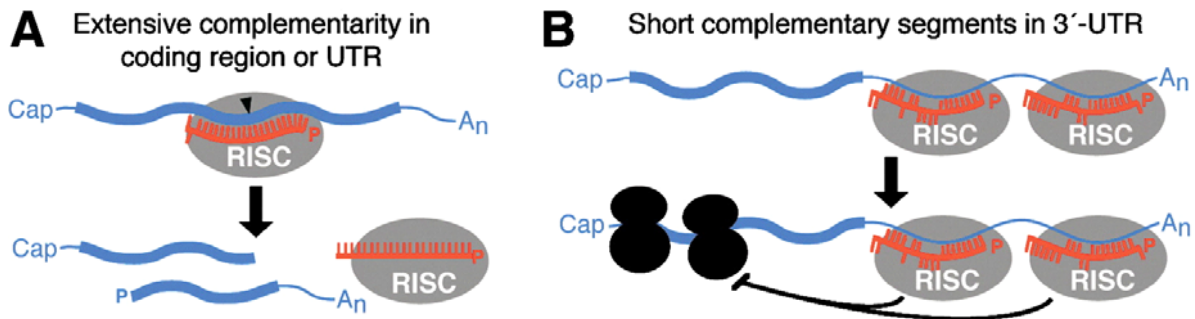


Fig. 3. MicroRNAs are post-transcriptional modulators of gene expression that either induce target mRNA cleavage (A) or translational inhibition (B). From Bartel 2004. Cell 116: 281-297.

The number of regulatory mRNA targets of vertebrate miRNAs has been estimated by identifying conserved complementarity to the miRNA seed sequences (nucleotide 2-7 of the miRNA), suggesting that ~30 % of the human genes may be miRNA targets (Lewis et al. 2005). Computational predictions in *Drosophila* provide evidence that a given miRNA has on average ~100 mRNA target sites in the fly, while another recent study reported that vertebrate miRNAs target ~200 mRNAs each, further supporting the notion that miRNAs can regulate the expression of a large fraction of the protein-coding genes in multicellular eukaryotes (Brennecke et al. 2005, Krek et al. 2005). Most recent reports indicate that miRNAs may not function as developmental switches, but rather play a role in maintaining tissue identity by conferring accuracy to gene-expression programs (Giraldez et al. 2005, Lim et al. 2005, Stark et al. 2005, Farh et al. 2005, Wienholds et al. 2005; Figs 1 and 4).

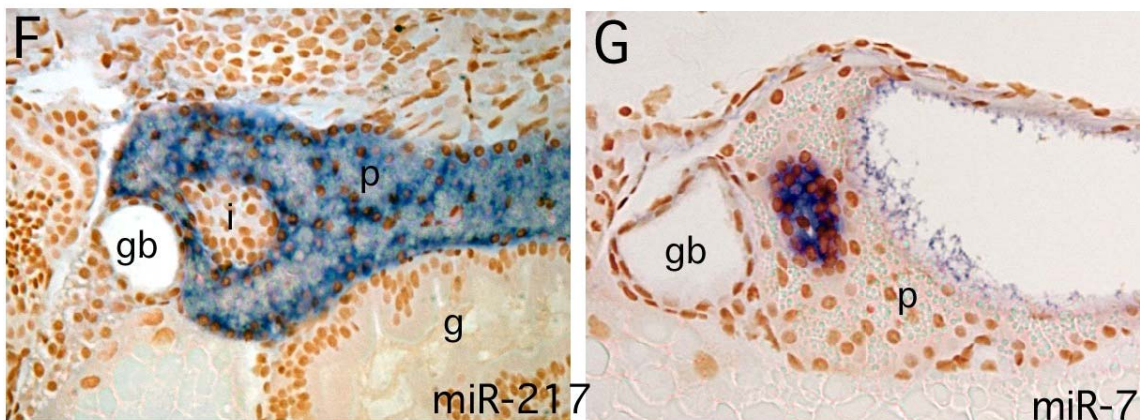


Fig. 4. Histological analysis of miRNA *in situ* expression in zebrafish pancreas at five days post-fertilization using LNA probes for miR-217 and miR-7. From Wienholds et al. 2005. Science 309: 310-311.

MicroRNAs in human disease

The expanding inventory of human miRNAs along with their highly diverse expression patterns and high number of potential target mRNAs suggest that miRNAs are involved in a wide variety of human diseases. One is spinal muscular atrophy (SMA), a paediatric neurodegenerative disease caused by reduced protein levels or loss-of-function mutations of the survival of motor neurons (SMN) gene (Paushkin et al. 2002). A mutation in the target site of miR-189 in the human SLITRK1 gene was recently shown to be associated with Tourette's syndrome (Abelson et al. 2005), while another recent study reported that the hepatitis C virus (HCV) RNA genome interacts with a host-cell microRNA, the liver-specific miR-122a, to facilitate its replication in the host (Jopling et al. 2005). Other diseases in which miRNAs or their processing machinery have been implicated, include fragile X mental retardation (FXMR) caused by absence of the fragile X mental retardation protein (FMRP) (Nelson et al. 2003, Jin et al. 2004) and DiGeorge syndrome (Landthaler et al. 2004).

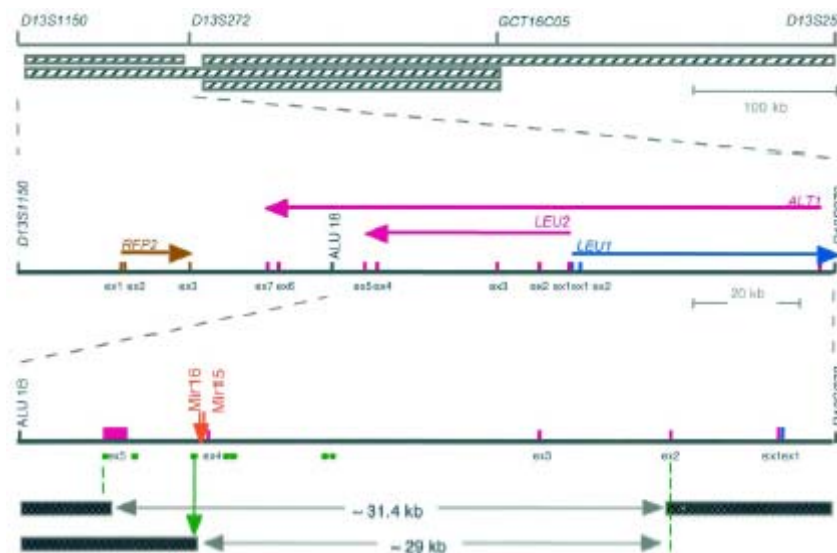


Fig. 5. The human microRNAs miR15 and miR16 are located at chromosome 13q14, a region deleted in more than half of B cell chronic lymphocytic leukemias (B-CLL). From Calin *et al.* 2002. PNAS 99: 15524-15529.

Perturbed miRNA expression patterns have been reported in many human cancers. For example, the human miRNA genes miR15a and miR16-1 are deleted or down-regulated in the majority of B-cell chronic lymphocytic leukemia (CLL) cases (Fig. 5), where a unique signature of 13 miRNA genes was recently shown to associate with prognosis and progression (Calin et al. 2002, Calin et al. 2005). The role of miRNAs in cancer is further supported by the fact that more than 50 % of the human miRNA genes are located in cancer-associated genomic regions or at fragile sites (Calin et al. 2004). Recently, systematic expression analysis of a diversity of human cancers revealed a general down-regulation of miRNAs in tumors compared to normal tissues (Lu et al. 2005). Interestingly, miRNA-based classification of poorly differentiated tumors was successful, whereas mRNA profiles were highly inaccurate when applied to the same samples. miRNAs have also been shown to be deregulated in lung cancer (Johnson et al. 2005) and colon cancer (Michael et al. 2004), while the miR-17-92 cluster, which is amplified in human B-cell lymphomas and miR-155 which is upregulated in Burkitt's lymphoma have

been reported as the first human miRNA oncogenes (Eis et al. 2005, He et al. 2005). Thus, human miRNAs would not only be highly useful as biomarkers for future cancer diagnostics, but are rapidly emerging as attractive targets for disease intervention by antisense oligonucleotide technologies.

LNA – a unique Danish invention with high potential in therapeutic miRNA silencing

LNAs comprise a new class of bicyclic high-affinity RNA analogues in which the furanose ring in the sugar-phosphate backbone is chemically locked in a RNA mimicking N-type (C3'-endo) conformation by the introduction of a 2'-O,4'-C methylene bridge (Koshkin et al., 1998). Several studies have demonstrated that LNA-modified oligonucleotides exhibit unprecedented thermal stability when hybridized with their RNA target molecules (Koshkin et al., 1998, Braasch and Corey, 2001; Kurreck, 2002). Consequently, an increase in melting temperature (T_m) of + 2-10 °C per monomer against complementary RNA compared to unmodified duplexes have been reported. Importantly, LNA incorporation generally improves mismatch discrimination compared to unmodified reference oligonucleotides. It should be underlined that LNA mediates high-affinity hybridization without compromising base pairing selectivity, and that the standard Watson-Crick base pairing rules are obeyed.

Structural studies of different LNA-RNA and LNA-DNA heteroduplexes based on NMR spectroscopy and X-ray crystallography have shown that LNA is an RNA mimic, which fits seamlessly into an A-type Watson-Crick duplex geometry (Petersen et al. 2000, Petersen et al. 2002, Nielsen et al. 2004) similar to that of dsRNA duplexes. Furthermore, in heteroduplexes between LNA oligonucleotides and their complementary DNA oligonucleotides, an overall shift from a B-type duplex towards an A-type duplex has been reported resulting in increased stability of the heteroduplexes. Another important observation is that LNA monomers are also able to twist the sugar conformation of flanking DNA nucleotides from an S-type (C2'-endo) towards an N-type sugar pucker in LNA-modified DNA oligonucleotides (Petersen et al., 2002; Nielsen et al., 2004).

The high thermal stability of short LNA oligonucleotides together with their improved mismatch discrimination has facilitated the design of highly accurate single nucleotide polymorphism (SNP) genotyping assays using allele-specific LNA probes (Jacobsen et al., 2002a; Jacobsen et al., 2002b, Mouritzen et al., 2003). LNA substituted oligonucleotides have also been used to increase the sensitivity and specificity in gene expression profiling by spotted oligonucleotide microarrays (Tolstrup et al., 2003) and more recently, in efficient isolation of intact poly(A)+RNA from lysed cell and tissue extracts by LNA oligo(T) affinity capture (Jacobsen et al., 2004). In addition, LNA oligonucleotides are readily transfected into cells using standard techniques, they are sequence-specific and non-toxic, and show improved nuclease resistance, which make them highly useful for potent and selective antisense-based gene silencing (Wahlestedt et al., 2000; Braasch et al., 2002; Fluiter et al., 2003). Hence, LNA-modified probes are uniquely suited for mimicking miRNA structures, and for miRNA targeting in vitro or in vivo.

Therapeutic intervention of disease-related microRNAs using LNA

The small size of miRNA genes makes it difficult to create loss-of-function mutants for functional analysis. Another potential problem is that many miRNA genes are present in several copies per genome occurring in different loci, which makes it even more difficult to obtain mutant phenotypes. An alternative approach to creating miRNA gene knock-outs has been reported by Hutvagner et al., (2004) and Leaman et al., (2005), in which 2'-O-methyl an-

tisense oligonucleotides were used as potent and irreversible inhibitors of siRNA and miRNA function in vitro and in vivo in *Drosophila* and *C. elegans*, thereby inducing a loss-of-function phenotype. This method was recently applied to mouse studies, by conjugating 2'-O-methyl antisense oligonucleotides complementary to four different miRNAs with cholesterol (so called antago-mirs) for silencing miRNAs in vivo (Krützfeldt et al., 2005).

Recent studies have reported that LNA-modified oligonucleotides can also mediate specific inhibition of miRNA function (Chan et al., 2005; Lecellier et al., 2005; Ørom et al., 2006). Using the well-characterized interaction between the *Drosophila melanogaster* bantam miRNA and its target gene *hid* as a model, Ørom et al., (2006) have described the efficacy and specificity of the LNA-based silencing method. LNA antimirs can readily inhibit exogenously introduced miRNAs with high specificity, and furthermore inhibit endogenous bantam in *Drosophila melanogaster* cells, leading to up-regulation of its cognate target protein *hid*. The method showed stoichiometric and reliable inhibition of the targeted miRNA and would thus be applicable to functional analysis of miRNAs and validation of putative target genes (Ørom et al., 2006). Since LNA antisense oligonucleotides have features that result in very high hybridization affinity towards complementary single stranded RNA without compromising specificity (Koshkin, 1998; Wahlestedt et al., 2000; Braasch and Corey, 2001) and furthermore, show improved antisense efficacy and higher T_m toward complementary RNA compared to 2'-O methyl oligonucleotides of the same sequence (Kurreck et al., 2002), LNAs can be used as effector molecules in highly potent and selective antisense-based gene therapy of disease-related microRNAs. Santaris Pharma is a world-leading expert in LNA antisense-based drugs, to which the company holds worldwide exclusive therapeutic rights. Besides an oligonucleotide drug for Chronic Lymphocytic Leukaemia in clinical phase I/IIa, Santaris has two other drugs against cancer under preclinical investigations.

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