

# Discovering the first microRNA-targeted drug

Morten Lindow<sup>1,2</sup> and Sakari Kauppinen<sup>2,3</sup>

<sup>1</sup>Department of Biology, The Bioinformatics Centre, University of Copenhagen, DK-2200 Copenhagen, Denmark

<sup>2</sup>Santaris Pharma, DK-2970 Hørsholm, Denmark

<sup>3</sup>Department of Health Science and Technology, Aalborg University Copenhagen, DK-2450 Copenhagen SV, Denmark

MicroRNAs (miRNAs) are important post-transcriptional regulators of nearly every biological process in the cell and play key roles in the pathogenesis of human disease. As a result, there are many drug discovery programs that focus on developing miRNA-based therapeutics. The most advanced of these programs targets the liver-expressed miRNA-122 using the locked nucleic acid (LNA)-modified antisense oligonucleotide miravirsen. Here, we describe the discovery of miravirsen, which is currently in phase 2 clinical trials for treatment of hepatitis C virus (HCV) infection.

## The discovery of microRNAs: From worm genetics to human disease

The first miRNA gene, *lin-4*, was discovered in *Caenorhabditis elegans* by Victor Ambros and Gary Ruvkun in 1993 (Fig. 1), and shown to control developmental timing in the worm by binding to partially complementary sites in the 3' untranslated region (UTR) of *lin-14* mRNA (Lee et al., 1993; Wightman et al., 1993). For several years *lin-4* was considered an oddity in worm genetics, until the discovery of a second, small regulatory RNA in the worm, called *let-7*, which was found to be highly conserved among animals, including humans (Pasquinelli et al., 2000; Reinhart et al., 2000). These seminal discoveries attracted the research laboratories of Victor Ambros, David Bartel, and Thomas Tuschl to search for similar RNAs in animals by small RNA cloning, which led to the discovery of numerous miRNAs in *C. elegans*, *Drosophila* embryos, and human HeLa cells (Fig. 1; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Soon after, the first studies linking miRNA dysregulation with human disease were published (Fig. 1). The human miRNA genes *mir-15a* and *mir-16-1* were found to be deleted or down-regulated in the majority of B cell chronic lymphocytic leukemia (CLL) cases (Calin et al., 2002). Furthermore, the miR-17-92 cluster, which is amplified in many cancers, including B cell lymphomas, and miR-155, which is over-expressed in hematological malignancies, were reported as the

first human oncogenic miRNAs (Eis et al., 2005; He et al., 2005; Costinean et al., 2006). Since then, 18,226 miRNAs have been annotated in animals, plants, and viruses, including 1,921 miRNAs encoded in the human genome (Kozomara and Griffiths-Jones, 2011). Indeed, miRNAs are predicted to repress a large fraction of all protein-coding genes and to participate in the regulation of almost every biological process in the cell (Kloosterman and Plasterk, 2006; Bushati and Cohen, 2007; Bartel, 2009; Friedman et al., 2009; Ambros, 2011). Moreover, recent work implies that miRNA dysregulation is frequently associated with the pathogenesis of human diseases (Gottwein and Cullen, 2008; Ventura and Jacks, 2009; Williams et al., 2009; Mendell and Olson, 2012), and has led to the discovery of several disease-implicated miRNAs that show promise as therapeutic targets (Stenvang et al., 2012; van Rooij et al., 2012).

## The first challenge: Developing the right tools

As the miRNA research field progressed, the first challenge was to develop and improve miRNA detection and functional analysis tools given the small size and sometimes low level of expression of different miRNAs. An important addition to the miRNA toolbox came from LNA (locked nucleic acid), a bicyclic high-affinity RNA analogue in which the ribose ring is chemically locked in an N-type (C3'-endo) conformation by the introduction of a 2'-O,4'-C methylene bridge (Koshkin et al., 1998). LNA-modified oligonucleotides possess high thermal stability when hybridized with their cognate mRNA target molecules (Koshkin et al., 1998; Braasch and Corey, 2001; Kurreck et al., 2002), and therefore appeared ideally suited for targeting of small RNAs, such as miRNAs. Thus, Sakari Kauppinen, who had been working with the development of LNA research tools since 2001, initiated a collaboration with the laboratories of Zoltán Havelda and József Burgyán to assess the utility of LNA probes in miRNA detection in plants. Indeed, this research team first reported the improved sensitivity and high specificity of LNA-modified oligonucleotide probes in detecting different plant miRNAs by small RNA Northern blot analysis (Válóczi et al., 2004) and in situ hybridization (Fig. 1; Válóczi et al., 2006). Encouraged by these findings, Kauppinen collaborated with

Correspondence to Sakari Kauppinen: sk@bio.aau.dk

Abbreviations used in this paper: HCV, hepatitis C virus; LNA, locked nucleic acid; miRNA, microRNA; PS, phosphorothioate; UTR, untranslated region.

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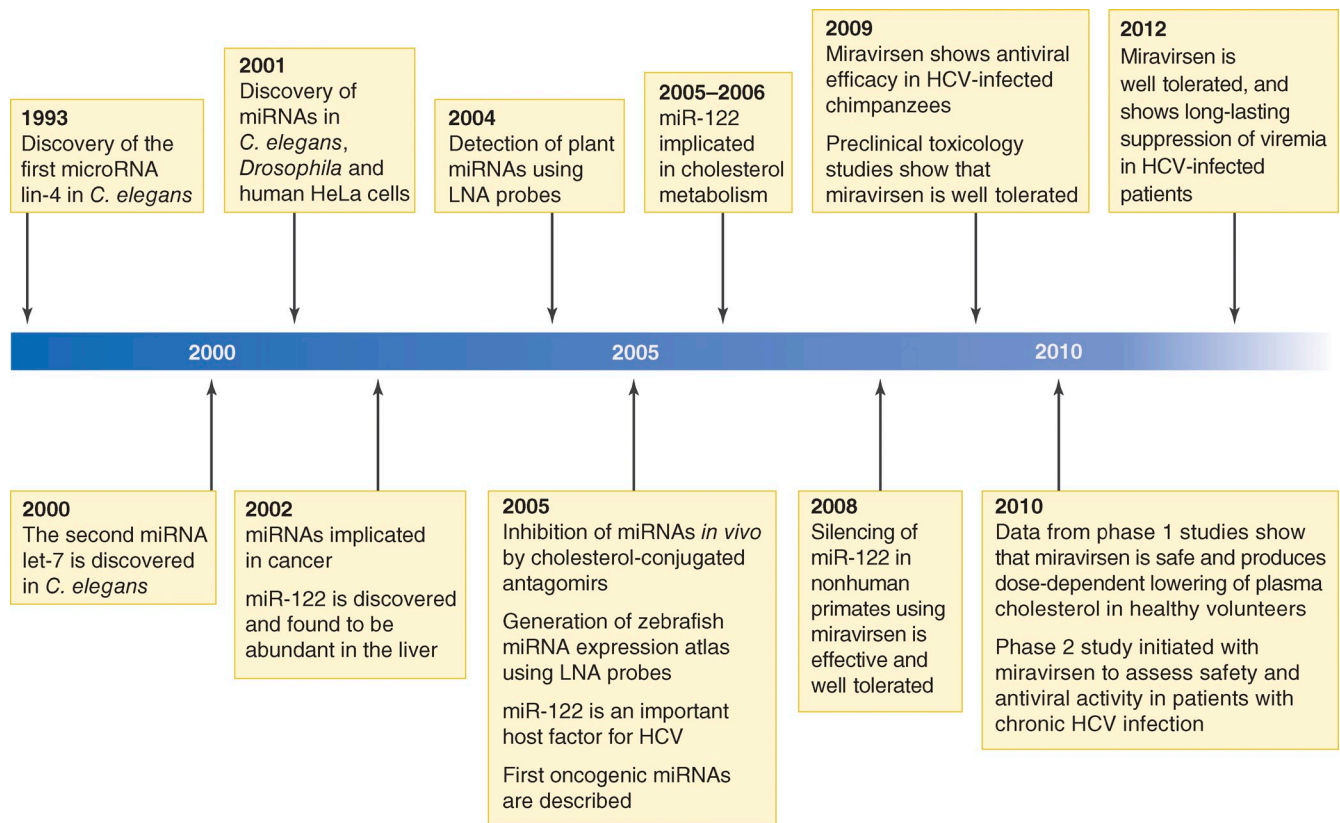


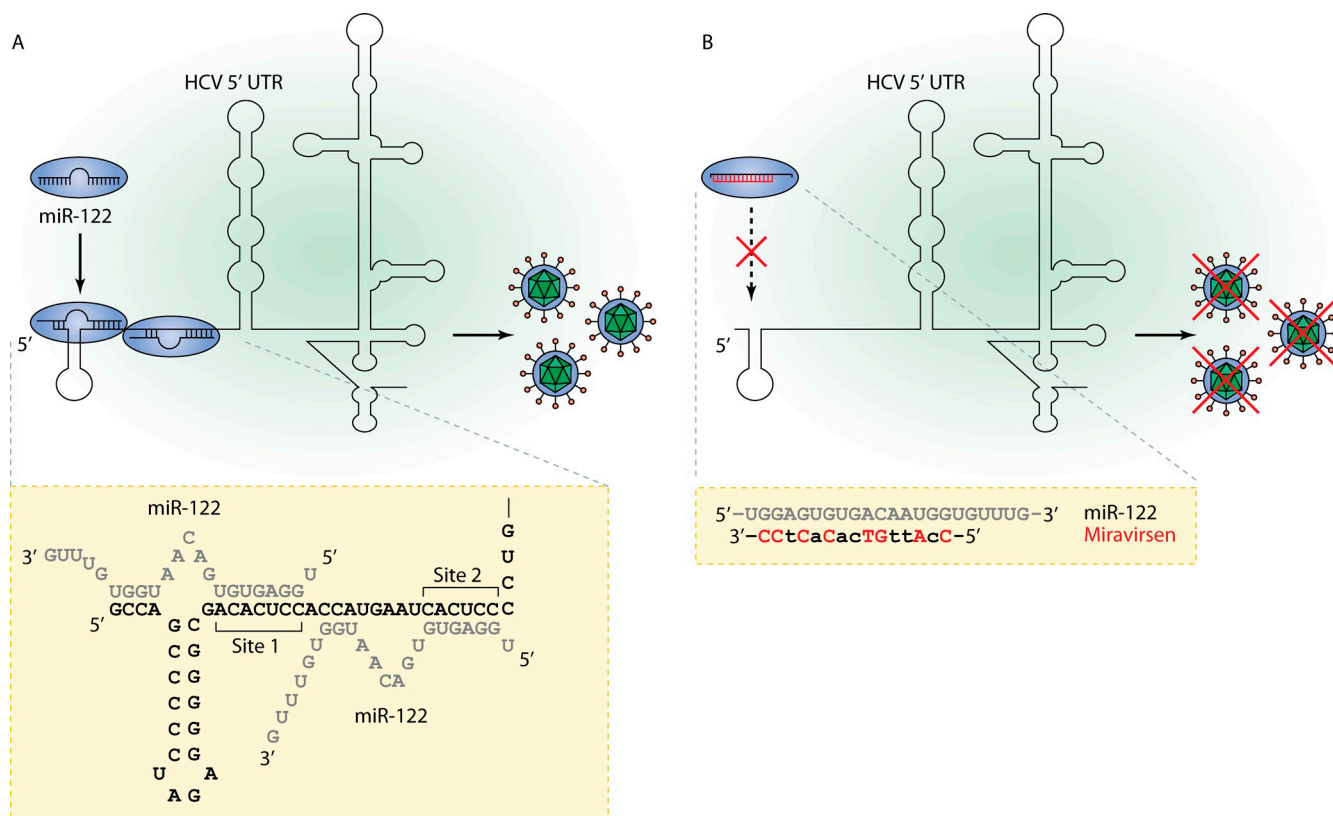
Figure 1. **Key findings in miRNA research and the discovery of the first miRNA-targeted drug, miravirsen.** The timeline indicates the key events from the discovery of the first microRNA, *lin-4*, in *C. elegans* to clinical testing of miravirsen in HCV-infected patients.

Ronald Plasterk, then at the Hubrecht Institute, who applied LNA probes in the detection of zebrafish miRNAs. In 2005, the Plasterk group reported the spatial and temporal expression patterns of 115 conserved vertebrate miRNAs that were determined by whole-mount in situ hybridizations in zebrafish embryos with LNA-modified DNA probes similar to those used in miRNA detection in plants (Fig. 1; Wienholds et al., 2005). In a follow-up study, Kloosterman et al. (2006) demonstrated the utility of LNA-based miRNA in situ hybridization in mouse embryos, whereas another report showed that LNA-modified antisense oligonucleotides, termed antimiRs, could be used to inhibit miRNA function in cultured cells (Ørom et al., 2006). From these studies, LNAs emerged as an important tool in miRNA research.

### The second challenge: Finding the right target

**microRNA-122: From zebrafish to therapeutic target in liver disease.** In 2005, several independent discoveries in the miRNA field led us to embark on the goal of developing the world's first microRNA therapeutic (Fig. 1). First, emerging evidence indicated that miRNA dysregulation was associated with the pathogenesis of human disease. Second, as described in the previous section, the successful use of short LNA probes with high melting temperatures in miRNA in situ detection and in inhibiting miRNA function in cells suggested that the LNA chemistry would enable the design of high affinity antimiR

oligonucleotides for pharmacological inhibition of disease-implicated miRNAs in vivo, provided that we could tackle the delivery issues. Third, cloning of tissue-specific miRNAs from mouse alongside the zebrafish miRNA expression atlas featured an animal miRNA named miR-122 (Lagos-Quintana et al., 2002; Wienholds et al., 2005) that caught our attention. Not only was miR-122 completely conserved in all vertebrates from zebrafish to man, but it also turned out to be highly abundant and expressed almost exclusively in the liver. These characteristics made miR-122 especially attractive for therapeutic targeting because lessons learned from three decades of research on phosphorothioate (PS)-modified antisense oligonucleotides had shown that they accumulated efficiently in the liver (Crooke, 2007). This work suggested that antimiR oligonucleotides with PS backbone modifications could similarly be delivered to the liver without additional conjugation or formulation chemistries. Finally, in a seminal study published in 2005, Peter Sarnow and his team reported that the liver-expressed miR-122 interacts with the HCV RNA by binding to two miR-122 seed sites located in the 5' UTR of the virus genome, leading to up-regulation of the viral RNA in infected liver cells (Fig. 2 A; Jopling et al., 2005). This implied that miR-122 was an important host factor for the virus and, hence, a potential therapeutic target for treatment of HCV infection, a leading cause of liver disease with over 170 million infected individuals worldwide. The idea of developing a miR-122-based antiviral therapy was especially appealing, due to the many limitations and side effects of the current standard of care HCV therapy.



**Figure 2. A model for interaction of miR-122 with the 5' UTR of the HCV RNA and miravirsens mechanism of action.** (A) The liver-expressed miR-122 is an important host factor for HCV and interacts with the 5' untranslated region (UTR) of the virus genome by binding to two miR-122 seed sites in association with Ago2. By forming an oligomeric miR-122-HCV complex, miR-122 protects the 5' UTR from nucleolytic degradation, thereby promoting viral RNA stability and propagation (Jopling et al., 2005; Machlin et al., 2011; Shimakami et al., 2012). (B) Miravirsens is a high-affinity 15-mer LNA-modified anti-miR oligonucleotide (red uppercase, LNA modifications; lowercase, DNA) that acts by sequestering mature miR-122, leading to inhibition of miR-122 function and thereby suppression of HCV.

### The third challenge: Identifying a promising lead compound

Our first goal was to obtain a fast proof-of-concept for targeting of miR-122 using an LNA-modified anti-miR oligonucleotide, and, thus, we chose a molecular design similar to that used in LNA probes for miRNA Northern blots and miRNA in situ hybridization, and adapted it for in vivo use by substituting the phosphodiester linkages with PS linkages. While this work was in progress in our laboratory, two studies reported on pharmacological inhibition of miR-122 in the mouse liver. Krützfeldt et al. (2005) described for the first time effective miR-122 silencing in mice using 3' cholesterol-conjugated, 2'-O-Me oligonucleotides, termed antagomirs, after three intravenous doses of 80 mg/kg antagomir-122, whereas Esau et al. (2006) used an unconjugated 2'-O-methoxyethyl-modified PS oligonucleotide and achieved inhibition of miR-122 in mice after four weeks of treatment by twice weekly intraperitoneal doses ranging from 12.5 to 75 mg/kg/dose. Notably, both studies implicated miR-122 as a modulator of hepatic lipid metabolism and reported a 30–40% decrease in plasma cholesterol levels in the treated mice, which thereby provided a highly useful biomarker for assessing anti-miR-122 activity in vivo. Our strategy for inhibiting miR-122 in the mouse liver turned out to be successful: a 16-mer LNA-modified anti-miR oligonucleotide reproduced the low cholesterol phenotype upon systemic delivery in mice (Elmén et al., 2008a).

In parallel with this work, we were exploring different LNA designs to improve the anti-miR drug potency. To this end, we synthesized a small library of LNA-modified anti-miRs, in which we incorporated several features that we considered as important in the design of potent miRNA inhibitors (Elmén et al., 2008b). First, the use of LNA enabled us to synthesize short oligonucleotides with high melting temperatures, and we therefore decided to truncate the anti-miR oligonucleotides from a fully complementary format to designs comprising 13-mer to 16-mer anti-miRs with a high melting temperature of at least 60°C. Second, the anti-miRs were all designed to target the 5' end of miR-122 due to the importance of the miRNA seed region (nucleotides 2–8) in miRNA target recognition (Krek et al., 2005; Lewis et al., 2005). Third, we excluded the nucleotide complementary to the first nucleotide in the miRNA sequence because structural studies had indicated that it was hidden in a separate binding pocket in the Argonaute protein (Ma et al., 2005; Parker et al., 2005). Fourth, to prevent possible slicing of the anti-miR by the RISC complex, we introduced LNA modifications at the site of passenger strand cleavage (Matranga et al., 2005; Leuschner et al., 2006). Finally, to enhance the pharmacokinetic properties and improve nuclease resistance, all anti-miR compounds were synthesized with a complete PS backbone and preferentially with LNA modifications at the 5' and 3' end of the anti-miR oligonucleotides (Elmén et al., 2008b).



Next, to establish a sensitive assay that would allow us to quickly screen our anti-miR inhibitor library in cultured cells, we cloned a perfect match miR-122 target site into the 3' UTR of the *Renilla* luciferase reporter gene. Using this miR-122 luciferase reporter assay as a workhorse, we found that inhibition of miR-122 function in cultured human Huh-7 hepatoma cells by the different LNA oligonucleotides was affinity dependent. Our screen identified a 15-mer LNA-modified anti-miR, named SPC3649, with a high melting temperature of 80°C, which mediated robust inhibition of miR-122 function in the liver cells when transfected at low nanomolar concentrations (Elmén et al., 2008b). Moreover, in close collaboration with the Sarnow laboratory at Stanford University, we found that SPC3649 was also the most potent inhibitor of HCV RNA accumulation in Huh-7 cells harboring the HCV-N replicon (Fig. 2 B; Elmén et al., 2008b). Finally, SPC3649 showed markedly improved efficiency in antagonizing miR-122 in mice compared with animals that were treated with either cholesterol-conjugated antagomir-122 or with other unconjugated anti-miR oligonucleotides (Krützfeldt et al., 2005; Esau et al., 2006; Elmén et al., 2008b). These experiments identified SPC3649 (later named miravirsen) as the lead candidate for the world's first miRNA-targeted drug.

#### **The fourth challenge: Testing in nonhuman primates**

An important step in the development of miravirsen was to ask whether it could mediate pharmacological inhibition of miR-122 in nonhuman primates. Collaborating with Matthew Lawrence and his team at RxGen, we performed a pharmacology study in African green monkeys at the research facilities on St. Kitts in the West Indies. Indeed, miravirsen's efficacy in mice translated very well to primates, as shown by monitoring plasma cholesterol levels in monkeys over time after administration of PBS-formulated miravirsen at doses ranging from 1 to 10 mg/kg with three intravenous injections over five days (Fig. 1). This treatment resulted in efficient sequestration of miR-122, leading to dose-dependent and long-lasting decrease of total plasma cholesterol that returned to baseline over a three-month period (Elmén et al., 2008b). We also found miravirsen to be well tolerated without any acute or subchronic toxicity in the monkeys. Indeed, these data showed for the first time that efficient antagonism of miR-122 can be achieved in nonhuman primates by simple delivery of unconjugated LNA-modified anti-miR oligonucleotides. Moreover, the safety profile in this study as well as those in subsequent preclinical toxicology studies in cynomolgus monkeys (Hildebrandt-Eriksen et al., 2012) were found to be very favorable. Highly encouraged by these findings, we were thus prepared to take the next steps toward the clinic.

**Miravirsen shows efficacy in HCV-infected chimpanzees.** Because miravirsen was a first-in-class inhibitor of a miRNA, we wanted to obtain proof-of-concept in an animal model of HCV before advancing it to clinical trials. Together with Robert Lanford at the Southwest Foundation of Biomedical Research we performed an efficacy study in the only validated animal model for HCV: chronically HCV-infected chimpanzee (Lanford et al., 2010). In this study, two chimpanzees

were administered with 12 weekly intravenous doses of 5 mg/kg and two with 12 doses of 1 mg/kg of miravirsen. A significant decline of the HCV viral titer was detected in the sera of the two chimpanzees in the 5 mg/kg miravirsen dose group with a maximum decrease of 2.6 orders of magnitude two weeks after last miravirsen dose, with no evidence of side effects in the treated animals. Importantly, no viral rebound was detected during the 12-wk treatment phase, which was consistent with the lack of adaptive mutations in the two miR-122 seed sites of the HCV 5' UTR, suggesting that miravirsen had a high barrier to viral resistance. These findings are in stark contrast to what has been observed with direct acting antivirals, where resistance and viral breakthrough in the worst cases develop already after 6–7 days of treatment (Cooper et al., 2009). In addition, the fact that the two critical miR-122 binding sites are completely conserved in all HCV genotypes implied that the antiviral effect of miravirsen on HCV would be genotype independent, which has recently been confirmed by another study (Li et al., 2011).

#### **The fifth challenge: Advancing miravirsen from bench to bedside**

The miR-122 inhibitor miravirsen is the first and to date only miRNA-targeted drug to have entered human clinical trials. Two phase 1 safety studies have been performed in healthy male volunteers: (1) a single ascending dose study with doses up to 12 mg/kg, and (2) a multiple ascending dose study with up to five doses of 5 mg/kg. Data from these studies demonstrated dose-dependent pharmacology as measured by lowering of plasma cholesterol, and showed that miravirsen was well tolerated with no dose-limiting toxicities (Hildebrandt-Eriksen et al., 2009). In 2010, Santaris Pharma initiated a phase 2a study to assess the safety and antiviral activity of miravirsen in treatment-naïve HCV patients (Janssen et al., 2011). In this study, 36 patients with chronic HCV genotype 1 infection were enrolled and randomized to three cohorts with nine miravirsen-treated and three placebo-treated subjects in each cohort. Treatment with miravirsen at doses of 3, 5, or 7 mg/kg as a total of 5 weekly subcutaneous injections over 29 days provided dose-dependent and long-lasting antiviral activity with a mean decrease of 2–3 logs from baseline HCV RNA levels ( $\log_{10}$  IU/ml). Furthermore, HCV RNA became undetectable in four out of nine patients in the high dose cohort (Janssen et al., 2011). The reported adverse events were infrequent and mostly mild, such as headache or coryza, and no serious adverse events or clinically significant changes in safety tests, vital signs, or electrocardiograms were observed (Janssen et al., 2011). Taken together, these data indicate that a four-week miravirsen monotherapy provides long-lasting suppression of viremia, has a high barrier to viral resistance, and is well tolerated in patients with chronic HCV infection.

So what began with the seminal discovery of the founding member of microRNAs, lin-4, in the worm just two decades ago, has now translated to clinical testing of the world's first miRNA-targeted drug in HCV-infected patients, thereby setting the stage for improved anti-HCV therapy. We believe that a large part of the success and the remarkable speed by which miravirsen has advanced from bench to bedside is due to the many

highly fruitful and agile collaborations between researchers in academia and the biotech industry with a shared vision of translating an exciting scientific discovery into a new therapy to improve human health. Thus, we hope that this story can serve as an inspiration and example for the benefit of both science and patients and look forward to seeing the next microRNA-based drug enter clinical development.

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