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MicroRNAs in common diseases and potential therapeutic applications

Louis M Tsai and Di Yu

Immunology and Inflammation Research Program, Garvan Institute of Medical Research, Sydney, New South Wales, Australia

SUMMARY

1. Evidence gathered in recent years has revealed microRNAs (miRNAs) fine-tune gene expression and play an important role in various cellular processes, including cell growth, differentiation, proliferation and apoptosis.

2. The present review summarizes current knowledge of miRNA pathways in the pathogenesis of cancer, cardiac diseases, neurodegenerative diseases, diabetes, autoimmune/inflammatory diseases and infection.

3. There is considerable potential to target miRNAs as a novel approach in the treatment of human diseases. Currently, miRNA-based therapies are being examined in both animal models and human clinical trials.

Key words: disease, microRNA, therapy.

INTRODUCTION

The 2006 Nobel Prize in Physiology or Medicine honoured the discovery that double-stranded RNA triggers the suppression of gene activity in a Watson–Crick base pairing-dependent manner, a process termed RNA interference (RNAi). Although both short interfering (si) RNA and microRNA (miRNA), two primary categories of small non-coding RNAs, occur naturally in plants and animals, miRNA functions primarily in animals.[1] Therefore, the scope of the present review is limited to a discussion of miRNA-mediated regulation in mammals with a focus on providing a comprehensive but succinct summary of the role of miRNAs in disease and therapy.

Correspondence: Di Yu, Immunology and Inflammation Research Program, Garvan Institute of Medical Research, Sydney, New South Wales 2010, Australia. Email: d.yu@garvan.org.au

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RNA SILENCING THROUGH THE miRNA PATHWAY

MiRNAs are often encoded within the introns of protein-coding genes, but can also be found as independent transcription units or in polycystronic clusters.[2] The miRNA genes are transcribed predominantly by RNA polymerase II into primary miRNAs (pri-miRNAs). These stem–loop transcripts are subsequently cleaved in the nucleus by the RNAse III enzyme Drosha. The approximately 65 nucleotide (nt) precursor miRNAs (pre-miRNAs) yielded are exported to the cytoplasm by Exportin 5 and further processed by a different RNAse III enzyme, namely Dicer. The resulting 19–25 double-stranded duplexes are unwound by a helicase and the mature miRNA strand is retained in the functional miRNA-induced silencing complex (miRISC), whereas the other strand is typically degraded. The miRISC also contains Argonaute proteins and other protein cofactors that facilitate the recognition of mRNAs by miRNAs and enable them to perform their suppressive function. The complementarity between the miRNA and mRNA tends to be imperfect in mammals, except for a region between residues 2 and 9 at the 5′ end of the miRNA known as the 'seed' region, which is usually completely complementary. The binding of mature miRNAs to target mRNAs, primarily within the 3′ untranslated region (UTR), mediates post-transcriptional repression of gene expression largely through translational inhibition and/or mRNA destabilization (Fig. 1; for a comprehensive review, see Carthew and Sontheimer[1]).

The current miRNA registry database (Release 13.0, March 2009; http://www.mirbase.org/) consists of 706 human miRNA genes, as well as 547 and 286 genes for mouse and rat, respectively.[3] Many new miRNA genes are being validated. Bioinformatic methods estimate that there are as many as 1000 miRNA genes within the human genome.[4] Individual miRNAs can control dozens to hundreds of target genes by the specificity of the seed sequence, as well as through other intrinsic mRNA characteristics, such as the adenylate uridylate (AU)-rich nucleotide composition near the miRNA-targeting site, proximity to sites for coexpressed miRNAs, proximity to residues pairing to miRNA nt 13–16, positioning within the 3′-UTR at least 15 nt from the stop codon and positioning away from the centre of long UTRs.[5] The prediction of miRNA target genes is an important yet challenging step in miRNA studies. Many algorithms have been designed to predict miRNA target genes based on base pairing in the seed region and thermodynamic stability of the miRNA–mRNA duplex. In addition, these algorithms frequently use extra criteria, including phylogenetic conservation

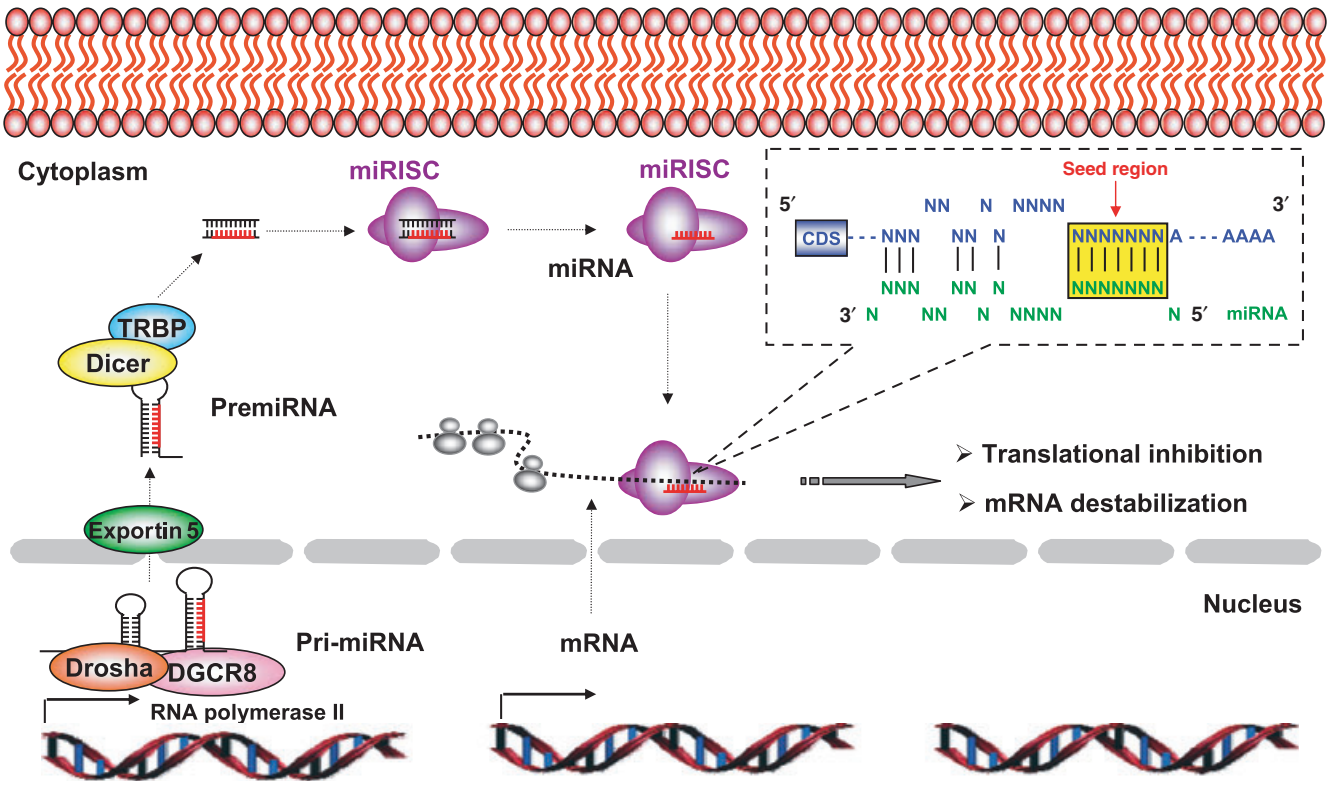

Fig. 1 Mammalian microRNA (miRNA) biogenesis and function. In the mammalian genome, RNA polymerase II transcribes miRNA-encoded genes into a long primary miRNA transcript (pri-miRNA). This pri-miRNA is processed into a precursor miRNA (premiRNA) by the nuclear RNase III enzyme Drosha partnered with DiGeorge syndrome critical region gene 8 (DGCR8). After being actively transported to the cytoplasm by exportin 5, the premiRNA is further processed by the RNase III enzyme Dicer and its cofactor TAR RNA binding protein (TRBP).[2] The miRNA-induced silencing complex (miRISC) recognizes the miRNA duplex and loads the functional strand of the duplex (mature miRNAs) onto the target mRNA. The base pairing between miRNA and mRNA is often imperfect, allowing for several mismatches, although the so-called 'seed' sequence of the miRNA (nucleotides 2–8 from 5′) usually matches perfectly. Once bound, the complex causes either inhibition of translation or targets the messenger for degradation. CDS, coding sequence.

of the seed region, the number of target sites in a single target and secondary mRNA structure at the miRNA target site. Several widely used programs are available online, such as TargetScan[6] (http://www.targetscan.org/), PicTar[7] (http://pictar.mdc-berlin.de/) and MiRanda (http://www.microrna.org/microrna/home.do).[8] For a complete summary of the tools used for predicting metazoan miRNA targets, authors are directed to a recent review by David Bartel.[9]

The miRNAs are one of many factors, including chromatin remodelling, transcription factors and splicing factors, that control gene expression in eukaryotic cells. It is now hypothesized that miRNAs regulate cellular functions by fine-tuning gene expression; recent analysis of the impact of miRNAs on protein output has substantiated this hypothesis by demonstrating that miRNA-induced gene repression is not absolute but, rather, occurs to a modest degree.[10,11] However, this fine-tuning function of miRNAs should not be underestimated. There are many examples where even one- or twofold changes in miRNA levels can significantly modulate cell physiological processes because these miRNAs co-ordinately repress multiple targets with related functions.[12]

MicroRNAs AND DISEASE

MicroRNAs and cancer

The abnormal levels of miRNAs in tumours have important pathogenic consequences and are commonly the consequence of the overexpression of tumour suppressor-targeting miRNAs or the down-regulation of oncogene-targeting miRNAs. One of the earliest pieces of evidence for the involvement of miRNAs in tumourigenesis came from the discovery that miR-15a and miR-16–1 are deleted or down-regulated in the majority of chronic lymphocytic leukaemia (CLL).[13,14] Both miRNAs negatively regulate anti-apoptotic Bcl2. The inverse correlation between miR-15a and miR-16–1 expression and Bcl2 expression in CLL provides an explanation for Bcl2 upregulation in most CLL cases, which exhibit a lack of Bcl-2 genomic translocation.[13] The region containing the miR-17–92 polycistron is often found amplified in human B cell lymphomas and enforced expression of the miR-17–92 cluster accelerates tumour development in the *Eμ-myc* transgenic mouse model of B cell lymphoma.[15] Cell cycle-regulating E2F transcription factors (i.e. E2F1, E2F2 and E2F3) and the pro-apoptotic molecules Bcl2 interacting mediator of cell death (Bim) and phosphatase and tensin homologue (PTEN) were verified as miR-17–92 targets.[16,17] Because miR-17–92 is itself upregulated by E2Fs and c-Myc, it operates in a complex feedback loop to control cell proliferation and apoptosis.[18] MiR-21 is another miRNA that is significantly upregulated in many tumours, including breast, lung, colon, prostate, pancreatic and stomach tumours.[19] The mechanism of action of miR-21 as an oncomiR, a term borrowed from 'oncogene', was explained by suppression of PTEN[20] and programmed cell death 4 (PDCD4).[21] The suppression of PDCD4 was suggested to mediate activator protein (AP)-1 activity in Harvey sarcoma virus (RAS) transformation.[21] One of the central pathways that

prevent tumourigenesis is the p53 tumour suppressor protein, which induces growth arrest, mediates DNA repair and promotes apoptosis. Conversely, mutations in the p53 pathway are found in nearly all cancers. Recently, miRNA joined the p53 regulatory network after the miR-34 family members were shown to be the direct transcriptional targets of p53.[22] Surprisingly, miR-34 activation recapitulates elements of p53 activity, including induction of cell cycle arrest and the promotion of apoptosis, whereas loss of miR-34 impairs p53-mediated cell death.[23] In addition, p53 itself is upregulated by miR-29 family members, which directly suppress phosphatidylinositol 3-kinase p85α and GTPase CDC42, two negative regulators of p53.[24] Conversely, p53 is suppressed by miR-125b.[25]

MicroRNAs and cardiac disease

One of the approaches used to investigate the role of miRNA in diseases is to block key enzymes in miRNA biogenesis. Using Cre-Lox technology, Dicer deleted in cardiac progenitors with Cre recombinase under control of the endogenous Nkx2.5 regulatory region leads to early embryonic lethality in mice.[26] If Cre recombinase is expressed under the control of α-myosin heavy chain (MHC) promoter, embryos could survive to birth. However, these neonates died within 4 days with symptoms of rapidly progressive dilated cardiomyopathy and heart failure.[27] These conditions may be the result of abnormal expression of cardiac contractile proteins and profound sarcomere disarray.[27] Importantly, decreased Dicer expression was observed in patients with end-stage dilated cardiomyopathy and failing hearts, whereas Dicer expression was increased in these hearts after left ventricle assist devices had been inserted to improve cardiac function.[27] The functions of individual miRNAs, particularly muscle-specific miR-1 and miR-133, have been investigated using genetically modified animals. Mice with targeted deletion of miR-1–2 developed ventricular septal defects, characterized by a hole in the wall between the left and right ventricles of the heart, as well as abnormal cardiac conduction and hyperplasmia.[26] In a separate study, overexpression of miR-1 in rat hearts had a prominent effect on the development of cardiac arrhythmia, coincident with the elevated levels of miR-1 expression from patients with coronary artery disease.[28] The miR-1 family consists of two miRNAs encoded by distinct but almost identical genes, designated *miR-1–1* and *miR-1–2*. The inability of the redundant miR-1–1 to compensate for the loss of miR-1–2,[26] together with the pathogenic effects of overexpression of miR-1,[28] reveals that precise regulation of miR-1 levels is crucial for the maintenance of normal cardiac function. Studies on molecular mechanisms suggested that the targets of miR-1 include heart and neural crest derivatives expressed 2 (Hand2), iroquois homeobox 5 (Irx5), gap junction protein, alpha 1 (GJA1; also named connexin 43) and K^+ channel subunit Kir2.1 (KCNJ2).[26,28] Residing within the same genomic cluster as miR-1 is another miRNA, namely miR-133, which has been observed to be decreased in both mouse and human models of cardiac hypertrophy.[29] Both targeted deletion and transgenic overexpression of miR-133a in mice resulted in ventricular septal defect and heart failure.[30] These phenotypes can be attributed, at least in part, to the dysregulation of serum response factor (SRF) and cyclin D2, both of which are specific targets of miR-133.[30] The interactive and synergistic control of cardiac function by a bicistronic *miR-1* and *miR-133a* cluster has been discussed in a recent review.[31] The cardiac-specific miR-208 is encoded by an intron of α-MHC. Mice deficient in miR-208 developed slight defects in heart physiology, but did not develop cardiac hypertrophy,[32] suggesting that miR-208 mediates the switch from expression of the heavy chain of α-myosin to that of β-myosin during stress or thyroid hormone-induced cardiac growth. This effect may be linked to repression of the thyroid hormone coregulator thyroid hormone receptor associated protein 1 (THRAP1), a transcriptional activator and suppressor.[32] In addition to cardiac- and/or muscle-specific miRNAs, the ubiquitously expressed miR-21 has been shown to contribute to myocardial diseases. Levels of miR-21 are selectively increased in fibroblasts of the failing heart,[33] enhancing interstitial fibrosis and cardiac hypertrophy through suppression of sprouty homologue 1 (SPRY1), which indirectly augments extracellular signal-regulated kinase–mitogen-activated protein kinase activity. Clinically, silencing miR-21 attenuates cardiac dysfunction and shows therapeutic efficacy.[33]

MicroRNAs and neurodegenerative diseases

Several studies have investigated mice with conditional knockout of Dicer in various post-mitotic neuronal cell types, including Prukinje cells, midbrain dopaminergic neurons and cortical and hippocampal neurons. These studies all demonstrated a progressive loss of neurons.[34–36] In these studies, miR-133b was specifically identified as an miRNA expressed in midbrain dopaminergic neurons, but deficient in midbrain tissue from patients with Parkinson's disease.[35] It was suggested that miR-133b regulated the maturation and function of dopaminergic neurons within a negative feedback circuit via suppression of paired-like homeodomain 3 (Pitx3).[35] In addition, miR-19, miR-101 and miR-130 have been found to directly target ataxin1 and coregulate its expression.[37] Because overexpression of ataxin1 with CAG repeats contributes to the severity of disease, the miRNA-mediated regulation of ataxin1 provides a new target for the modulation of the pathogenesis of neurodegenerative diseases sensitive to protein dosage.[37]

MicroRNAs and diabetes

Initially, miR-375 was identified among several miRNAs expressed selectively in pancreatic endocrine cell lines.[38] In addition, studies *ex vivo* revealed that overexpression of miR-375 resulted in suppressed glucose-stimulated insulin secretion, whereas inhibition of miR-375 enhanced insulin secretion, both through the suppression by miR-375 of myotrophin and pyruvate dehydrogenase kinase, isozyme 1 (PDK1).[38,39] Mice lacking miR-375 are hyperglycaemic: they exhibit increased total numbers of pancreatic α-cells, fasting and fed plasma glucagon levels and increased gluconeogenesis and hepatic glucose output, whereas pancreatic β-cell mass is decreased as a result of impaired proliferation.[40]

MicroRNAs and autoimmune/inflammatory diseases

The first evidence describing a role for the miRNA machinery in preventing systemic autoimmunity used a strain of mice homozygous for a hypomorphic variant of the *roquin* gene (sanroque mice) that develop high titres of autoantibodies and a lupus-like systemic autoimmunity, as well as autoimmune diabetes against a genetically susceptible background.[41] Roquin is a newly discovered component involved in miRNA-mediated regulation. Hypomorphic mutant Roquin in sanroque mice led to impaired miRNA activity. Such

impairment resulted in decreased miR-101/103-mediated mRNA decay of the inducible T cell costimulator (Icos); the higher Icos level promoted lymphoproliferation.[42] Mice subjected to T cell-specific Dicer or Drosha deletion developed colitis and lung and liver inflammation from 4 months of age, with a > 50% reduction in numbers of regulatory T (Treg) cells. These cells are a specialized subpopulation of T cells that act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens.[43,44] Furthermore, generation of Treg-specific Dicer or Drosha knockout mice revealed a phenotype mimicking patients suffering from a severe and rapidly fatal autoimmune disorder known as immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome.[43,45,46] Lymphoproliferation has also been linked to the miRNA 17–92 cluster. Overexpression of the miRNA 17–92 cluster, frequently amplified in lymphoma, was demonstrated to drive lymphoproliferation and a lupus-like disease through the abnormal downregualtion of PTEN and Bim transcripts, two important negative regulators of T cell activation, proliferation and survival.[17]

MicroRNAs and infection

Viral miRNAs are expressed predominantly by herpes viruses, a family of DNA viruses.[47] Many viral miRNAs target viral mRNAs, as exemplified by miR-BART2. This miRNA is encoded by Epstein–Barr virus (EBV) and it inhibits EBV DNA polymerase BALF5.[48] Other examples are miR-H2–3p, miR-H6, miR-H3 and miR-H4 in the herpes simplex virus 1 (HSV-1), which provide a candidate mechanism regulating viral proteins between latency and productive replication.[47] The polyma virus SV40 encodes MiR-S1 with a perfect complementarity to SV40 T-antigen mRNA. The miR-S1-mediated downregulation of SV40 T-antigen facilitates the virus's evasion of cytotoxic T cells.[49] Therefore, miR-S1 helps to protect SV40-infected cells from immune detection. Viral miRNAs also modulate cellular mRNAs to enhance infection. For example, miR-UL112-1 expressed by human cytomegalovirus (HCMV) has been shown to target major histocompatibility complex class I polypeptide-related sequence B (MICB) and to inhibit NK cell killing of virus-infected cells.[50] Conversely, viral miRNA targets the apoptosis pathway to promote the survival of infected cells, which is evidenced by miR-BART5, expressed by EBV to inhibit pro-apoptotic protein p53-upregulated modulator of apoptosis (PUMA).[51] For more examples and a comprehensive summary of the function of viral miRNAs, readers are directed to a recent review.[47] Although RNA viruses, including retroviruses and flaviviruses, have not been reported to express miRNAs to date,[47] an interesting hypothesis proposed by two studies suggests that the Tat protein expressed by human immunodeficiency virus (HIV) inhibits the activity of Dicer in infected cells.[52] The infection-dependent downregulation of Dicer lowers miRNAs, including miR-17–5p and miR-20a, which potentially target the histone acetylase p300/CBP associated factor (PCAF), an important cofactor for Tat-mediated transactivation of gene expression. This mechanism should enhance HIV transcription, which is supported by increased HIV replication after experimental knockdown of Dicer and Drosha.[53] Cellular miRNAs can actively regulate viral infection. For example, miR-24 and miR-93 target viral large protein (L protein) and phosphoprotein (P protein) genes of vesicular stomatitis virus (VSV) to limit viral replication, which explains the susceptibility of Dicer-deficient mice to VSV infection.[54] Similarly, miR-32 targets the open reading frame two of primate foamy virus type 1 (PFV-1) and thus restricts the accumulation of the virus in human cells.[55] In contrast, endogenous miR-122, specifically produced by liver cells, binds to the 5′-UTR of hepatitis C virus (HCV) and, surprisingly, promotes viral RNA replication.[56] Examination of liver biopsies has shown decreased levels of miR-122 in individuals with HCV who responded poorly to interferon (IFN) therapy.[57] Interestingly, IFN-β, an antivirus cytokine secreted by the immune system, leads to a significant reduction in the expression of the liver-specific miR-122, which implies mammalian organisms, through the IFN system, use cellular miRNAs to combat viral infections.[58] A cluster of miRNAs enriched in resting $CD4^+$ T cells, such as miR-28, miR-125b, miR-150, miR-223 and miR-382, targets 3′ ends of HIV-1 mRNAs and promotes the latency of HIV-1 in resting primary $CD4^+$ T cells.[59] Considering that RNAi is a strategy used by plants and lower animals to defend against infection, it is likely that more reports will appear implicating host-derived miRNAs in defence against infectious agents. For a more extensive review on the reciprocal interaction between hosts and pathogens through miRNA pathways, readers are directed to the paper by Corbeau.[60]

MicroRNAs and therapy

Based on the link between abnormal miRNA expression and diseases, two major miRNA-based therapeutic strategies are: (i) to restore the expression of the miRNA reduced in diseases; and (ii) conversely, blunting overexpressed miRNA.

It has been known for several years that miRNAs and siRNAs share the same RNA-induced silencing complex (RISC) machinery to act on mRNAs.[1] Therefore, many of the lessons learned from siRNA-based therapeutics can be applied to enahnce miRNA expression by either transient delivery of miRNA mimics or the stable expression of miRNA. Similar to synthetic siRNAs, the major obstacle for miRNA mimic-based therapy is delivery *in vivo*. Many formulations, including cholesterol conjugation, aptamer conjugation, liposome/lipoplex and antibody–protamine fusion, have been designed to promote *in vivo* delivery efficacy and have shown promise in various cases (for a review, see de Fougerolles *et al.*[61]). In addition, miRNAs can be expressed through the processing of pri-miRNA transcripts driven by Pol II promoters or through short hairpin (sh) RNAs driven by Pol III promoters. The former is more versatile and flexible due to current inducible and/or tissue-specific expression systems driven by Pol II. The review by Wiznerowicz *et al.*[62] not only describes many expression systems for RNAi, but also discusses their applications. Adversely, such overexpression of miRNA could oversaturate endogenous small RNA pathways, as shown by the experiment of sustained high-level shRNA expression in livers of adult mice leading to serious hepatocellular toxicity and fatality.[63] This saturation effect was explained by the overloading of exportin-5 function, which mediates the nuclear export of premiRNAs and shRNAs (Fig. 1).[64] The risk of sustained expression means that inducible and/or tissue-specific expression of miRNA is a more attractive option in the future. Another concern for miRNA-based therapy is the off-target effect. As discussed before, one miRNA can control dozens to hundreds of target genes. Thus, careful design is necessary to narrow the target effect to the pathway of interest and to minimize side-effects on unrelated pathways, particularly those with housekeeping functions.

Artificial antisense oligonucleotides complementary to the miRNA sequence are designated antimiRs. Many chemical modifications have been designed to enhance miRNA inhibition, of which the method of locked nucleic acids (LNA) has exhibited unprecedented thermal stability with miRNA, high self-stability, low toxicity and good aqueous solubility *in vivo*.[65] The LNA is a bicyclic high-affinity RNA analogue, with the furanose ring in the sugar-phosphate backbone chemically locked in an RNA-mimicking N-type (C3′-endo) conformation by the introduction of a 2′-*O*,4′-C methylene bridge. Liver-expressed miR-122 is implicated in cholesterol and lipid metabolism.[66,67] The LNA antimiR-122 achieved efficient silencing of miR-122 in monkeys and led to a long-lasting and reversible decrease in total plasma cholesterol without any evidence of LNA-associated toxicities or histopathological changes.[68] This is the most readily applied example of miRNA-based therapy. One alternative inhibitor for miRNAs is the 'miRNA sponge'. These sponges are transcripts expressed from strong promoters containing multiple, tandem binding sites to an miRNA of interest that compete with natural miRNA targets for miRNA binding.[69] This method has been validated by experiments demonstrating bone marrow reconstitution with haematopoietic stem cells stably overexpressing the miR-223 target sequence (miR-223 sponge) to phenocopy the genetic miR-223 knockout mouse.[70]

Many biotech firms are advancing miRNA-based therapeutics to the clinic, including Asuragen (http://www.asuragen.com/), Crogen Phamaceuticals (http://crogenpharmaceuticals.com/), Miragen Therapeutics (http://www.miragentherapeutics.com/), Regulus Therapeutics (http://www.regulusrx.com/), Rosetta Genomics (http://www.rosettagenomics.com/) and Santaris Pharma (http://www.santaris.com/) A recent review discusses the prospects of RNAi therapeutics.[71]

CONCLUDING REMARKS

Since the first miRNA was identified in *Caenorhabditis elegans* in 1993,[72] there has been an explosive increase in our understanding of the mechanism of miRNA-mediated gene regulation, their role in disease and their application as new therapeutics. It is impossible to list all the important discoveries on miRNAs in such a short review. As our understanding of miRNA continues to evolve,[73] prospects for miRNA-based therapy will improve. In 2008, Santaris announced that it had commenced a clinical trial for an LNA-based antisense molecule targeting miR-122 for the treatment of hepatitis C, the first miRNA-based therapeutic to be trialled in humans (http://www.santaris.com/filemanager/items/spc3649_news_release_280508.pdf).

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