

# Anti-miRNA oligonucleotides (AMOs): ammunition to target miRNAs implicated in human disease?

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*MicroRNAs (miRNAs) are endogenous 19–25 nucleotide RNAs that have recently emerged as a novel class of important gene-regulatory molecules involved in many critical developmental and cellular functions. miRNAs have been implicated in the pathogenesis of several human diseases, such as neurodegenerative disorders, cancer, and more recently in viral and metabolic diseases. Unraveling the roles of miRNAs in cellular processes linked*

*to human diseases will lead to novel opportunities for the regulation of protein function and will help to evaluate their potential for therapeutic intervention. Approaches to interfere with miRNA function in vitro and in vivo based on synthetic anti-miRNA oligonucleotides (AMOs) are discussed in this review.*

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## Introduction

MicroRNAs (miRNAs) are a family of short noncoding regulatory RNA molecules expressed in a variety of different cell types from several animal species, ranging from *Caenorhabditis elegans* to humans, as well as in plants.<sup>1,2</sup> The miRNA pathway serves as an important post-transcriptional regulation mechanism<sup>3</sup> and the potential importance of miRNAs in pathologically significant pathways is increasingly appreciated. In order to investigate the potential causative roles of dysregulated miRNA processes in human disease, synthetic, chemically modified oligonucleotides to target miRNAs or their mRNA targets are proving to be powerful tools and may eventually find application in a new therapeutic mechanism.

## miRNA and their potential role in diseases

The biogenesis of miRNAs is a multistep process (Figure 1).<sup>4</sup> A primary miRNA transcript (pri-miRNA),<sup>5</sup> which is frequently synthesized from intronic regions of protein-coding RNA polymerase II transcripts,<sup>6,7</sup> is first processed by a protein complex containing the double-strand (ds)-specific ribonuclease Droscha in the nucleus to produce a hairpin intermediate of ~70 nucleotide (nt).<sup>8</sup> This precursor miRNA (pre-miRNA) is subsequently transported by Exportin-5/Ran-GTP<sup>9,10</sup> to the cytoplasm where it is cleaved by another dsRNA specific ribonuclease, Dicer,<sup>11,12</sup> into miRNA duplexes. After strand

separation of the duplexes, the mature single-stranded miRNA is incorporated into an RNA-induced silencing complex (RISC)-like ribonucleoprotein particle (miRNP).<sup>13–15</sup> This complex inhibits translation or, depending on the degree of Watson-Crick complementarity, induces degradation of target mRNAs.<sup>16</sup>

The international miRNA Registry database<sup>17</sup> contains more than 300 verified and putative homologues of human miRNA sequences. The increasing number and diversity of miRNAs suggests that they play a role in the regulation of many genes in key pathways in a wide variety of cellular processes such as cell cycle control, apoptosis,<sup>18</sup> haematopoiesis,<sup>2,19</sup> adipocyte differentiation<sup>20</sup> and insulin secretion.<sup>21</sup> There is a growing number of reports that link miRNAs to the regulation of pathways associated with human diseases such as cancer,<sup>22</sup> neurological diseases<sup>23</sup> and most recently also with viral<sup>24</sup> and metabolic diseases.<sup>21</sup> As yet, however, there is not conclusive evidence that causatively links the malfunction of a miRNA or a miRNA target site to the development of a human disease.

Modified synthetic anti-miRNA oligonucleotides (AMOs) are useful tools in specifically inhibiting individual miRNAs, thereby helping to unravel the function of miRNAs and their targets. Similar to antisense-based oligonucleotides (ASOs), AMOs may contribute to the prioritization of pharmaceutical targets and have the potential to eventually progress into a new class of therapeutic agents.

We review here literature that associates specific miRNAs with disease and reports of how AMOs have been used to help elucidate miRNA function.

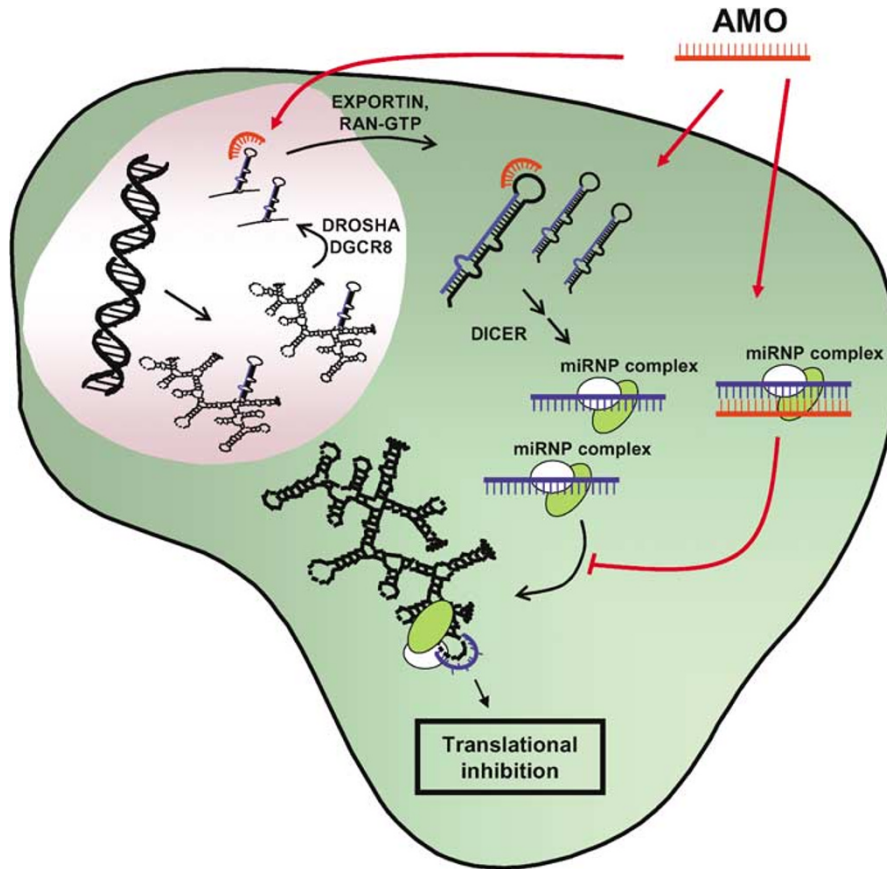
## miRNAs and cancer

Cancer is a genetic disease in which mutational and/or epigenetic changes in a genome leads to stepwise

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**Figure 1** Interference with the miRNA pathway using synthetic oligonucleotides. Inhibition of miRNA activity may be achieved by introducing anti-miRNA oligonucleotides (AMOs) fully complementary to the pri-miRNA, the pre-miRNA or the mature miRNA.

deregulation of cell proliferation and cell death mechanisms. Evidence is emerging that particular miRNAs may play a role in human cancer pathogenesis. For example, deletions or mutations in genes that code for miRNA tumor suppressors might lead to loss of a miRNA or miRNA cluster, and thereby contribute to inappropriate stabilization of oncogenes.<sup>25,26</sup> The results of a recent large-scale miRNA study suggest that 50% of miRNA genes are frequently located in cancer-associated genomic regions or fragile sites.<sup>27</sup> The genes encoding mir-15 and mir-16 are located at chromosome 13q14, a region that is deleted in the majority of B-cell chronic lymphocytic leukemias (B-CLL),<sup>28</sup> and in other cancers such as mantle cell lymphoma and prostate cancer.<sup>29</sup> Interestingly, none of the protein-coding genes in this region were found to cause B-CLL,<sup>30</sup> suggesting that mir-15 and mir-16 may possibly function as tumor suppressors. MiRNAs miR-143 and miR-145 display significant downregulation in colonic adenocarcinoma samples compared to matched normal mucosa tissues.<sup>31</sup> Putative mRNA targets of these miRNAs include several genes that have been implicated in oncogenesis such as RAF1 kinase, G-protein  $\gamma$  7 and tumor-suppressing subfragment candidate 1, although molecular interaction of these genes with their putative miRNA counterparts *in vivo* remains to be proven.

Johnson *et al.*<sup>32</sup> identified RAS, an oncogene that is overexpressed or mutated in many human cancers, as one of the genes regulated by several members of the let-

7 miRNA family. Levels of let-7a and let-7c were found to inversely correlate with RAS protein levels in matched diseased and healthy lung tissues. This correlation was experimentally confirmed in demonstrating that overexpression of a dsRNA that mimicked the let-7a precursor led to reduction of RAS whereas siRNA mediated inhibition of let-7 caused elevation of RAS in cell culture, suggesting that members of the let-7 miRNA family may safeguard against accelerated RAS expression. These findings are also consistent with earlier studies indicating that multiple let-7 genes are located in genomic regions frequently deleted in cancer patients.<sup>27</sup> Furthermore, reduced expression of let-7 is associated with shorter postoperative survival in lung cancer patients.<sup>33</sup>

The expression of miRNA-155, which is encoded by the BIC gene, was found to be elevated in children with Burkitt lymphoma.<sup>34</sup> BIC cooperates with the oncogene c-Myc in lymphomagenesis and erythroleukemogenesis.<sup>35</sup> Recently, c-Myc itself has been proposed to directly activate a cluster of six miRNAs, two of them negatively regulating the transcription factor E2F1, suggesting a new mechanism by which c-Myc tightly controls cell proliferation in a cell-type specific manner.<sup>36</sup> In another recent study,<sup>37</sup> an miRNA cluster was suggested to act as an oncogene. The mir-17-92 polycistron cluster, which is located on a DNA fragment that is amplified in human B-cell lymphomas promoted tumor development when overexpressed in a mouse B-cell lymphoma model.

## Viral miRNAs and human miRNAs targeted against viral genes

In small-sized viral genomes, miRNAs offer an efficient means to specifically inactivate host cell defense factors compared to virally encoded proteins. Several recent reports describe miRNAs cloned from a variety of viruses such as herpes viruses<sup>38,39</sup> and human immunodeficiency virus 1 (HIV-1).<sup>40</sup> Pfeffer *et al.*<sup>24</sup> identified five miRNAs in Epstein-Barr virus (EBV) through cloning of RNA from latently infected Burkitt's lymphoma cells. The same group<sup>39</sup> also found nine miRNAs encoded by human cytomegalovirus (HCMV) and, simultaneously, with Cai *et al.*<sup>38</sup> found 11 miRNAs from Kaposi's sarcoma-associated virus (KSHV). The cloned herpes virus-derived miRNAs are thought to target both viral as well as host genes. In some instances, the complementarity to the putative target mRNA is near perfect, presumably leading to target mRNA degradation. This was demonstrated for miRNAs derived from the circular dsDNA simian virus 40 (SV40), which are perfectly complementary to the early viral mRNA coding for T antigen and accumulate in the late stages during infection.<sup>41</sup> The reduced expression of this nonstructural protein renders the infected cells less sensitive to lysis by cytotoxic T cells.

Computational methods predict the occurrence of miRNAs in other dsDNA viruses such as herpes simplex virus 1 and 2, variola and vaccinia virus, molluscum contagiosum virus, human adenoviruses of the subgenus A, B, and D, and BK virus.<sup>39</sup> The same authors also predicted the occurrence of miRNAs in the genome of the smaller single-stranded RNA viruses, measles virus and yellow fever virus, although in the latter case this was not confirmed by cloning. Omoto *et al.*<sup>40,42</sup> identified a miRNA expressed from the HIV-1 nef-gene of AIDS patients who are long-term disease nonprogressors, and which encodes a miRNA that may suppress both Nef function and HIV-1 virulence through RNA silencing. Further putative HIV-1-derived miRNAs targeted against the T cell receptors CD4 and CD28, and several interleukins were proposed in a recent computational study.<sup>43</sup> Mammalian cells lack the ability to process viral dsRNA into siRNAs for subsequent destruction of the pathogen's genomic or messenger RNA.<sup>44</sup> Instead, human miRNAs may provide an antiviral defense, as postulated recently by Lecellier *et al.* Working with human cells lines infected with the retrovirus primate foamy virus 1 (PFV-1), they observed that blocking miR-32 with a complementary, modified oligonucleotide leads to near doubling of the PFV-1 replication rate. Sequence analysis of the genomes of 8 viruses from different families suggests that this mechanism might represent a widespread feature of host-virus interactions.<sup>45</sup>

In yet another twist to the interplay between host cell and virus, Lecellier *et al.*<sup>45</sup> also demonstrated that PFV-1 expresses a protein factor, Tas, which suppresses miRNA-directed functions in mammalian cells and also displays antisilencing activities in plants. HIV-1 has evolved a similar suppressor of RNA silencing, the Tat protein which besides its transactivating function inactivates human Dicer. Another adenovirus-derived inhibitor of RNA silencing has also been described.<sup>46</sup> The approximately 160-nt VA1 noncoding RNA is highly structured, and is expressed in adenovirus-infected cells.

It potently inhibits RNA silencing induced by shRNAs or human miRNA precursors, but does not affect RNA interference by synthetic siRNAs. RNA ligands that are naturally selected to bind protein targets specifically are referred to as aptamers.<sup>47</sup> In this case, inhibition is likely due to direct binding of Dicer as well as competition for the Exportin 5 nuclear export factor.

## miRNAs in metabolic diseases

Two recent publications have described particular miRNAs as potential therapeutic targets for the treatment of diabetes and obesity. In both studies, the respective mRNA targets were predicted and subsequently experimentally validated in cellular systems. The pancreatic islet-specific mir-375 was found to modulate glucose-stimulated insulin secretion and exocytosis, by blocking the expression of myotrophin, a protein associated with neuronal secretion.<sup>21</sup> In a similar manner, Esau *et al.* suggested a potential implication of miRNAs in the maturation of human adipocytes. Modified oligonucleotide complementary to miR-143 effectively suppressed adipocyte differentiation by modulation of its putative target ERK5, a protein previously known to be implicated in MAP kinase signaling pathways, but which had not yet directly been linked to adipocyte differentiation.<sup>20</sup>

## miRNPs in neurological and developmental disorders

Fragile X syndrome was probably the first human disease to be linked to a dysfunctioning of an miRNA pathway. The gene responsible for fragile X syndrome, *fmr1*, is inactivated through expansion of a CGG triplet repeat, methylation of which silences expression of the encoded protein, FMRP (fragile X mental retardation protein). FMRP is involved in synaptic plasticity and dendritic development.<sup>48</sup> The *Drosophila* homolog of FMRP, dFMR1, coimmunoprecipitates with several RISC components, among them AGO2 and VIG. Moreover, dFMR1 interacts with Dicer as well as miRNAs and exogenously added siRNAs.<sup>49,50</sup> This was also confirmed for mammalian FMRP *in vivo*.<sup>51</sup>

Loss or mutation of survival of motor neuron protein (SMN), a component of an miRNA containing ribonucleoprotein (RNP) complex, is thought to be the cause of spinal muscular atrophy (SMA), a disease characterized by the progressive degeneration of motor neurons.<sup>23</sup> SMN binds to Gemin3 in a RNP complex also consisting of Gemin4, eIF2C2 and various miRNAs.<sup>52</sup> This complex, which may be representative for the core of human RISC, was shown to cleave RNA substrates complementary to its constituent miRNA.<sup>13</sup>

Dostie *et al.*<sup>23</sup> have cloned a miRNA from Weri human retinoblastoma cells with a potential genetic link to two other neurological diseases, namely early onset parkinsonism (Waisman syndrome) and X-linked mental retardation (MRX3). Precursors to miR-224 and a second putative miRNA were found within an EST coding for the epsilon subunit of the GABA A receptor, whose genetic locus had previously been implicated in these diseases.

Numerous miRNAs are involved in developmental regulation of gene expression in model organisms.<sup>1</sup> DiGeorge syndrome is a rare congenital disease whose symptoms vary but include heart defects and characteristic facial features. It is caused by a large deletion from chromosome 22 and two recent studies revealed that *dgcr8*, a gene located within the deleted region, encodes a protein which interacts with Drosha during the processing of primary miRNA transcripts.<sup>53,54</sup> How this loss of activity relates to DiGeorge syndrome has not been described in detail, however.

## Targeting miRNA regulation with oligonucleotides

Oligonucleotide analogs are the natural choice of therapeutic class to correct the aberrant activity of any miRNA-mRNA interaction, which contributes causally to a disease.

Where a deletion or a loss-of-function mutation is present in the miRNA gene itself,<sup>27,55</sup> a therapeutic approach could entail exogenous delivery of corrective synthetic miRNAs in the form of (siRNA-like) dsRNA. This principle was first demonstrated *in vitro* by Zeng *et al.*,<sup>56</sup> who showed in a model system that partially complementary siRNAs can inhibit target mRNAs by miRNA-like translational inhibition. Given the vulnerability of unmodified dsRNA to nucleases *in vivo*, this class of compound could probably only be used in privileged local environments, for example in the central nervous system.<sup>57</sup>

If a disease phenotype is derived from abnormal inhibition of mRNAs caused by, for example, excessive expression of miRNAs, AMOs complementary to either the mature miRNA or its precursors<sup>58</sup> can be designed (Figure 1). The earliest report of miRNA inhibition using AMOs describes the microinjection of DNA oligonucleotides of the same length and complementary to the target miRNA into *Drosophila* embryos.<sup>59</sup> Unmodified DNA oligonucleotides were later found to be ineffective as

inhibitors of let-7 miRNA in *C. elegans*,<sup>60</sup> consistent with the known instability of unmodified DNA *in vivo*. Fully chemically modified oligonucleotides have previously been shown to be effective inhibitors of both coding and noncoding RNAs *in vitro* and *in vivo* (vide infra) and some of them such as an 20-mer phosphorodiamidate morpholino oligomer targeting c-Myc are currently under investigation in human clinical trials.<sup>61</sup> The most important property of such oligonucleotides is specificity and high binding affinity to RNA. From years of antisense research, a number of nucleoside modifications emerged, which yield increased binding affinity for RNA. In particular, the addition of chemical groups to the 2'-hydroxyl group has been rather fruitful<sup>62</sup> and a number of corresponding oligonucleotide derivatives are being pursued in clinical development and, therefore, may also be applicable as therapeutic AMOs. The following discussion is restricted to those types of modified oligonucleotides that have already been used as AMOs (Figure 2).

### 2'-O-Methyl AMOs

The 2'-O-methyl-group (OMe) is one of the oldest, simplest and most often used modifications to oligonucleotides. The methyl group contributes a limited amount of nuclease resistance, and improves binding affinity to RNA compared to unmodified sequences. Fully-modified OMe-oligonucleotides have been used to correct aberrant exon splicing in cells,<sup>63</sup> and mixed backbone OMe/DNA hybrid antisense oligonucleotides are also being pursued in clinical studies.<sup>64</sup> Hutvagner *et al.*<sup>60</sup> successfully demonstrated inhibition of let-7 function in HeLa cells, as well as *C. elegans* larvae, using 31-mer OMe-AMOs. Specificity of the interaction was confirmed using an unrelated RNA species with close sequence homology. The affinity between the OMe-oligonucleotide and a miRNA in a targeted RISC complex was approximately 40-fold stronger than with a fully complementary mRNA, suggesting that the protein components of the RISC complex greatly enhanced the interaction beyond mere hybridization. In

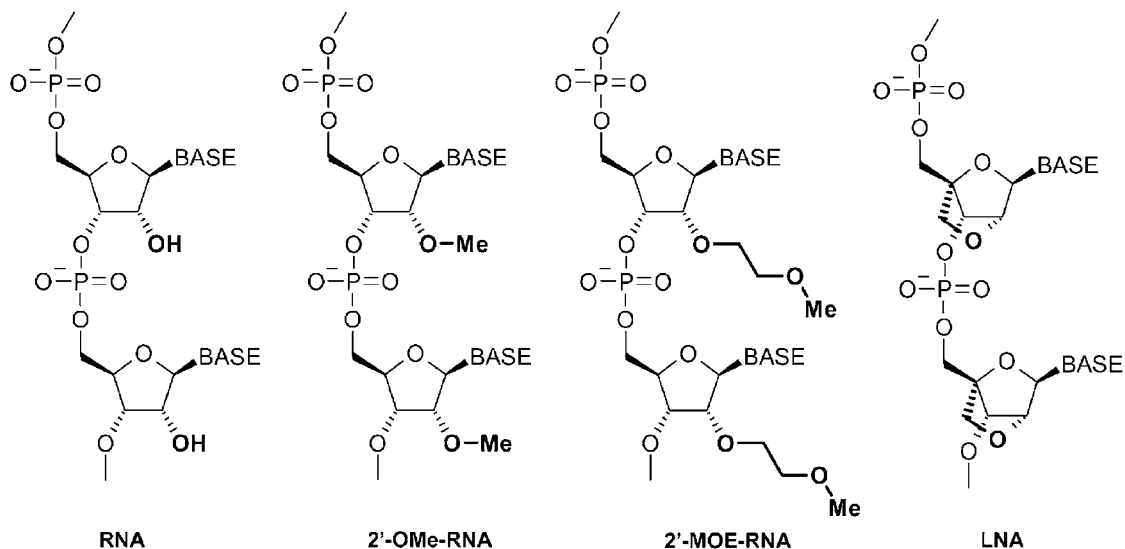


Figure 2 Chemically modified oligonucleotide analogs that have been used as anti-miRNA inhibitors (AMOs).

two other reports, OMe-AMOs were reported to abrogate siRNA or miRNA function in cellular assays. Meister *et al.*<sup>44</sup> showed that 24-mer OMe-AMOs but not natural DNA oligonucleotides could specifically abrogate miRNA function in cellular extracts as well as in cultured HeLa cells. Cheng *et al.*<sup>65</sup> applied a library of Me-AMOs to human cancer cell lines to screen for miRNAs potentially involved in cell growth and apoptosis. As an alternative to direct blockage of a particular miRNA, Lee *et al.*<sup>58</sup> recently suggested Me-AMOs directed against the loop region of a miRNA precursor as tools to determine miRNA function *in vivo*.

### 2'-O-Methoxyethyl AMOs

2'-O-Methoxyethyl (MOE)-modified oligonucleotides have higher affinity and specificity to RNA than their OMe-analogs.<sup>66</sup> They have been used effectively as fully modified oligonucleotides targeted to re-direct mRNA splicing,<sup>67</sup> and also as inhibitors of protein translation.<sup>68</sup> MOE-ASOs represent an increasing proportion of modified oligonucleotides in clinical trials.<sup>69</sup> Concerning their application in miRNA research, Esau *et al.*<sup>20</sup> transfected separately MOE-AMOs targeting 86 human miRNAs into cultured human preadipocytes to address the role of miRNAs in adipocyte differentiation. By following gene expression profiles of five marker genes they found miR-143 to be involved in this process through regulation of ERK5 protein levels. Treatment of adipocytes with a MOE-AMO complementary to miR-143 effectively inhibited the differentiation process, whereas negative controls were inactive. Furthermore, Northern blot analysis of cell lysates indicated that MOE-AMO treatment had also decreased levels of miR-143, presumably by inhibition of processing of the pri-miRNA by Drosha.

### Locked nucleic acid AMOs

Locked nucleic acid (LNA)-modified oligonucleotides are distinctive 2'-O-modified RNA in which the 2'-O-oxygen is bridged to the 4'-position via a methylene linker to form a rigid bicycle, locked into a C3'-endo (RNA) sugar conformation.<sup>70</sup> The LNA modification leads to the thermodynamically strongest duplex formation with complementary RNA known. Consequently, a biological activity is often attained with very short LNA oligonucleotides. For example, an 8 nt fully-modified LNA oligomer complementary to a structural loop inhibited 50% of self-splicing of group I introns from rRNA genes in pathogenic organisms whereas DNA and RNA oligonucleotides were ineffective.<sup>71</sup> Short fully-modified LNA oligonucleotides designed against telomerase were active in cellular assays, compared to mismatched negative controls.<sup>72</sup> Furthermore, LNAs display excellent mismatch discrimination. Mouritzen *et al.*<sup>73</sup> showed single-nucleotide specificity against complementary DNA using fully modified 12 nt LNA probes coupled to glass slides during the development of a microarray used to probe samples for single-nucleotide polymorphisms (SNPs) associated with human dysmetabolic syndrome. LNA oligonucleotides have not been tested in humans, but results in rodents are promising.<sup>74</sup> LNAs were stable and showed a serum decay and tissue distribution similar to that of phosphorothioate oligonucleotides. A fully complementary LNA oligonucleotide

targeted to murine POLR2A protein was effective using continuous administration at 2.5 mg/kg with no associated liver or kidney toxicity.

LNA oligonucleotides have been used as northern probes to detect miRNAs as mixed backbone oligonucleotides.<sup>75</sup> Excellent selectivity and sensitivity – at least 10-fold greater than with DNA – was obtained and single-nucleotide selectivity shown with a mismatch in the middle of the sequences. Mixed LNA/DNA AMOs potentially abolished miR-32 function in PFV-1 infected HeLa cells and led to the accumulation of viral mRNA, resulting in increased production of viral progeny.<sup>45</sup> Recently, Chan *et al.*<sup>76</sup> have successfully applied 2'-O-methyl- and DNA/LNA-mixed oligonucleotides to specifically knockdown miR-21 in order to investigate the potential contribution of this miRNA in the regulation of apoptosis-associated genes in glioblastoma cell lines.

## Conclusions

Although the precise cellular functions of most mammalian miRNAs are still unknown, increasing evidence suggests that miRNAs regulate many biological processes associated with human disease. However, critical questions remain as to the number of existing miRNAs, how many mRNA targets are regulated by a particular miRNA and how many miRNAs control a particular mRNA target.<sup>77–79</sup> An increasing number of highly sophisticated bioinformatics methods is being developed to identify additional putative miRNAs and their predicted miRNA target genes.<sup>79–81</sup> In addition, the systematic application of genomics technologies such as expression profiling<sup>82,83</sup> in combination with loss- and gain-of-function studies in cellular models might contribute to a better understanding of miRNA controlled cellular networks and may lead to new ways of intervention in human diseases. Without doubt, AMOs have the potential to become powerful tools in interfering with miRNA pathways thereby contributing to the dissection of their functions and their putative role in human disease processes.

The promise of antisense therapeutics has yet to be fulfilled. The regulatory approval of the first generation oligonucleotide Vitravene for CMV retinitis was not followed by a rush of further approvals, yet there are still many modified ASOs in clinical trials, especially in the area of cancer treatment.<sup>69</sup> Recently, siRNAs have reached clinical development, and the first proof of concept studies have been described, involving the local injection of dsRNA reagents into the eye.<sup>84,85</sup> Although, the prospects for AMO therapeutics are ultimately linked to the success of these oligonucleotide-based drugs, the principal question at this point remains: will miRNAs make good therapeutic targets?

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