Therapeutic targeting of microRNAs: current status and future challenges

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Abstract | MicroRNAs (miRNAs) are evolutionarily conserved small non-coding RNAs that have crucial roles in regulating gene expression. Increasing evidence supports a role for miRNAs in many human diseases, including cancer and autoimmune disorders. The function of miRNAs can be efficiently and specifically inhibited by chemically modified antisense oligonucleotides, supporting their potential as targets for the development of novel therapies for several diseases. In this Review we summarize our current knowledge of the design and performance of chemically modified miRNA-targeting antisense oligonucleotides, discuss various *in vivo* delivery strategies and analyse ongoing challenges to ensure the specificity and efficacy of therapeutic oligonucleotides *in vivo*. Finally, we review current progress on the clinical development of miRNA-targeting therapeutics.

More than a decade has passed since the discovery in *Caenorhabditis elegans* that double-stranded RNA (dsRNA) can induce potent and specific gene silencing, a phenomenon termed RNA interference (RNAi)[1]. Since then, a large body of work has demonstrated that RNAi is a well-conserved process that functions in several species, including mammals. Whereas RNAi in *C. elegans* is induced by endogenous long dsRNAs, in mammalian cells the inhibitory capability of RNA was initially demonstrated by the experimental introduction of small 21-nucleotide RNAs with perfect sequence complementarity to target mRNA transcripts[2]. The resulting search for endogenous RNAi triggers in mammalian cells led to the discovery of many small RNA species, the major classes of which are small non-coding RNAs (microRNAs; miRNAs), endogenous small interfering RNAs (siRNAs) and Piwi-interacting RNAs[3–6].

miRNAs are ~21–23-nucleotide single-stranded RNAs (ssRNAs) that have crucial roles in almost every aspect of biology, including embryonic development and the host response to pathogens. Increasing evidence suggests that miRNAs also contribute to a spectrum of human diseases, especially cancer. The first evidence for such a role came from the observation that miRNAs are frequently located in fragile regions and deleted sites in human cancer genomes[7,8]. Since then, many miRNAs have been reported to be closely associated not only with cancer development[9] but also with a number of other human conditions, including viral infections, cardiovascular diseases[10] and inflammatory diseases[11]. The importance of miRNA function and dysfunction in various human diseases thus suggested that modulation of miRNA expression may serve as a novel therapeutic modality for such diseases.

Various chemically modified oligonucleotides have been shown to efficiently block miRNA function *in vitro*[12–14], and many molecules have shown efficacy in preclinical animal models[15–17]. Recent advances have accelerated the clinical development of therapeutic oligonucleotides, and the first miRNA-targeting therapeutic is in now in clinical trials for hepatitis C virus (HCV) infection, fuelling hope for the success of this novel class of disease-modifying drugs.

In this Review we first briefly summarize the mechanisms of miRNA biogenesis and function, then evaluate current progress and key challenges in various miRNA-targeting strategies. Finally, we assess potential approaches to improve the design and performance of miRNA-targeting reagents *in vivo*. We hope this Review will prompt new ideas for the design of next-generation miRNA-targeting therapies with better *in vivo* target specificity and improved pharmacodynamic and pharmacokinetic (PK/PD) properties.

Biogenesis and function of miRNAs in mammals

miRNA genes are usually transcribed from RNA polymerase II promoters and then processed into mature miRNAs through canonical or non-canonical miRNA biogenesis pathways (FIG. 1). During canonical miRNA biogenesis, the primary miRNA (pri-miRNA) hairpin is digested to precursor miRNA (pre-miRNA) by Drosha, a member of the RNase III family. Non-canonical miRNA biogenesis

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doi:10.1038/nrd4359
Published online 11 July 2014

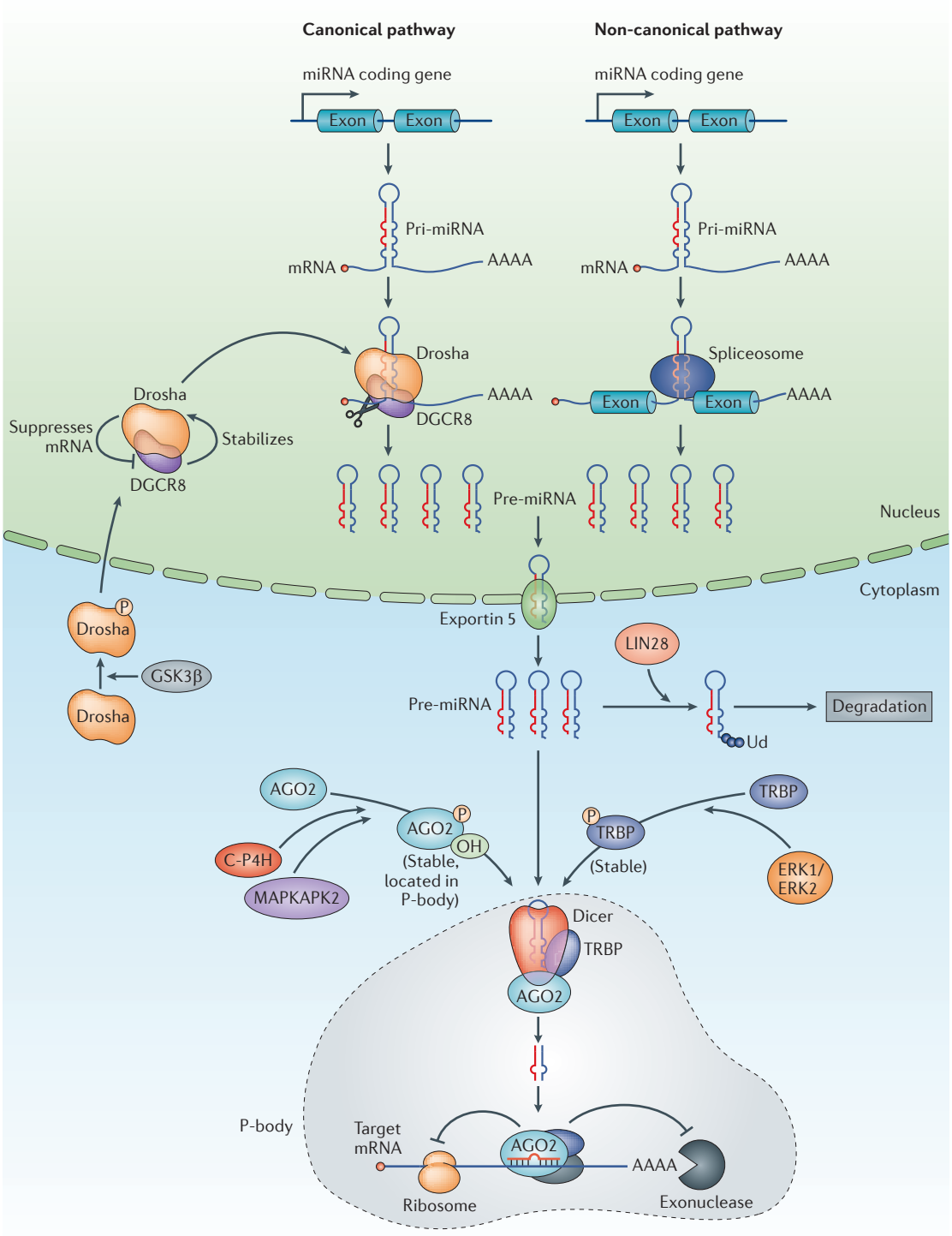

Figure 1 | **Canonical and non-canonical miRNA biogenesis pathways.** In the canonical pathway, microRNAs (miRNAs) are typically transcribed by RNA polymerase II to produce primary miRNA (pri-miRNA) hairpins, which are then processed by the Drosha–DGCR8 (DiGeorge syndrome critical region 8) complex to generate precursor miRNAs (pre-miRNAs). These molecules are transported by exportin 5 into the cytoplasm, where they are further processed by Dicer–TRBP (TAR RNA-binding protein 2) and loaded into Argonaute 2 (AGO2)-containing RNA-induced silencing complexes (RISCs) to suppress downstream target gene expression. miRNAs are also produced though non-canonical pathways, such as spliceosome-dependent mechanisms, as shown here. The miRNA biogenesis pathway is a tightly regulated process. For example, Drosha is dependent on phosphorylation by glycogen synthase kinase 3β (GSK3β) for proper nuclear localization[168]; Drosha regulates DGCR8 expression by suppressing *DGCR8* mRNA[20]; DGCR8 stabilizes Drosha protein[20]; AGO2 is hydroxylated by C-P4H[169] and phosphorylated by MAPK-activated protein kinase 2 (MAPKAPK2)[170], which stabilizes the protein and regulates its localization to processing bodies (P-bodies); and TRBP is stabilized by extracellular signal-regulated kinase 1 (ERK1) or ERK2 phosphorylation[25]. miRNAs themselves are regulated by a number of modifications, including uridylation (Ud)[171].

differs at this step in that pre-miRNAs are generated by mRNA splicing machinery, circumventing the requirement for Drosha-mediated digestion in the nucleus. In both pathways, the pre-miRNAs are exported to the cytoplasm via the nuclear export protein exportin 5 and further processed by a second RNase III enzyme, Dicer. The mature double-stranded miRNAs are then loaded into a functional ribonucleoprotein complex called the RNA-induced silencing complex (RISC), which serves as the catalytic engine for miRNA-mediated post-transcriptional gene silencing. RISC consists of multiple protein factors, and Argonaute proteins are the key catalytic enzymes within the complex. Argonaute proteins bind miRNAs and are essential for their downstream gene-regulatory mechanisms to regulate mRNA degradation and protein expression[18].

miRNA biogenesis is tightly controlled at multiple steps (FIG. 1). The majority of miRNA genes lie within intronic regions of coding genes and their expression is thus subject to the same types of transcriptional control as other cellular genes. Transcriptional regulation has been proposed to be the major mechanism controlling tissue- and cell type-specific expression of miRNAs[19]. The catalytic activity of Drosha and Dicer is also highly regulated, mainly through their ribonucleoprotein binding partners DiGeorge syndrome critical region 8 (DGCR8) and TAR RNA-binding protein 2 (TRBP), respectively, but also via other accessory protein factors such as LIN28, p68 (also known as DDX5) and p72 (also known as DDX17)[19]. Binding of DGCR8 to the central domain of Drosha helps to stabilize the enzyme complex, but excessive levels of DGCR8 have been reported to compromise Drosha activity[20–22]. Furthermore, Drosha can reduce DGCR8 expression by cleaving the hairpin structures contained in *DGCR8* mRNA[20,21]. Accumulation of Dicer is also dependent on its binding partner, as the protein is destabilized when TRBP expression is low[23,24]. The stability of TRBP itself is regulated by the mitogen-activated protein kinase (MAPK)–extracellular signal-regulated kinase (ERK) signalling pathway[25].

Once mature miRNAs are loaded into the RISC, the ribonucleoprotein complexes are able to bind to and regulate the expression of their target mRNAs. Binding of the miRNA-induced silencing complex (miRISC) to mRNA is mediated by a sequence of 2–8 nucleotides, known as the seed region, at the 5′ end of the mature miRNA[26]. Early studies of the *C. elegans* miRNA Lin-4 showed that miRNAs acted through translational repression[27,28]. However, it is now thought that miRNAs may act through several additional mechanisms, including inhibition of translation initiation[29], inhibition of translation post-initiation[30–32] and induction of mRNA destabilization and decay[33,34] (FIG. 2). In mammalian cells, mRNA destabilization is thought to be the dominant mode of action of miRNAs, possibly involving P-body proteins. P-bodies are also known as cytoplasmic processing bodies, which are enriched with enzymes and other proteins involved in mRNA degradation and sequestration from translational machinery. P-body components, such as GW182 (also known as TNRC6A)[35,36], mRNA-decapping enzyme 1 (DCP1), DCP2 (REF. 37) and the ATP-dependent RNA helicase p54 (also known as RCK and DDX6)[38], have been found to physically interact with Argonaute proteins and are essential for miRNA-mediated gene repression. It is also worth noting that each miRNA can regulate multiple target mRNAs simultaneously[39]. For some miRNAs, the targets are components of a single pathway[40–42], which suggests that miRNAs could be used to manipulate the activity of an entire pathway rather than the components alone. One such example is miR-17 family miRNAs, which target components of the transforming growth factor-β (TGFβ) signalling pathway, such as TGFβ receptors, SMADs and the downstream effector gene cyclin-dependent kinase inhibitor 1A (*CDKN1A*; which encodes p21), as well as several other genes[43,44]. Other examples include the targeting of tumour suppressor genes by miR-21 (REF. 45) and the targeting of key cell proliferation pathways by let-7 family miRNAs[46].

Targeting miRNAs

The realization that many miRNAs have crucial roles in basic biological processes and that dysregulation of miRNAs is common in human disease has led to considerable interest in the therapeutic targeting of miRNAs. To date, three main approaches have been taken: expression vectors (miRNA sponges), small-molecule inhibitors and antisense oligonucleotides (ASOs) (FIG. 3).

Vector-based strategies rely on the expression of mRNAs containing multiple artificial miRNA-binding sites, which act as decoys or 'sponges'[47]. Overexpression of the mRNA-specific sponges selectively sequesters endogenous miRNAs and thus allows expression of the target mRNAs. Although sponges have been widely used to investigate miRNA function *in vitro*, their utility *in vivo* has thus far been limited to transgenic animals in which the sponge mRNA is overexpressed in target tissues[48]. Interestingly, it seems that some large non-coding RNAs could serve as natural sponges to regulate cellular miRNA availability and lead to upregulation of downstream target genes[49–51].

Approaches that are based on small molecules are also being developed to manipulate miRNA expression and function. These approaches generally rely on reporter-based assay systems for compound library screening and have identified small molecules that could specifically inhibit miRNA expression, such as azobenzene (which affects miR-21 expression)[52] and several diverse compounds that inhibit miR-122 (REF. 53). The modes of action of these small molecules are mainly through transcriptional regulation of targeted miRNAs rather than inhibition of target recognition by these miRNAs. However, their therapeutic potential is rather limited owing to their high EC_{50} (effector concentration for half-maximum response) values, which are in the micromolar range, and the lack of information on direct targets.

Considerably more attention has been paid to ASO technology, particularly to those ASOs that target miRNAs directly (anti-miRs) to specifically inhibit miRNA function. Anti-miRs bind with high complementarity to miRISCs, thereby blocking their binding to endogenous

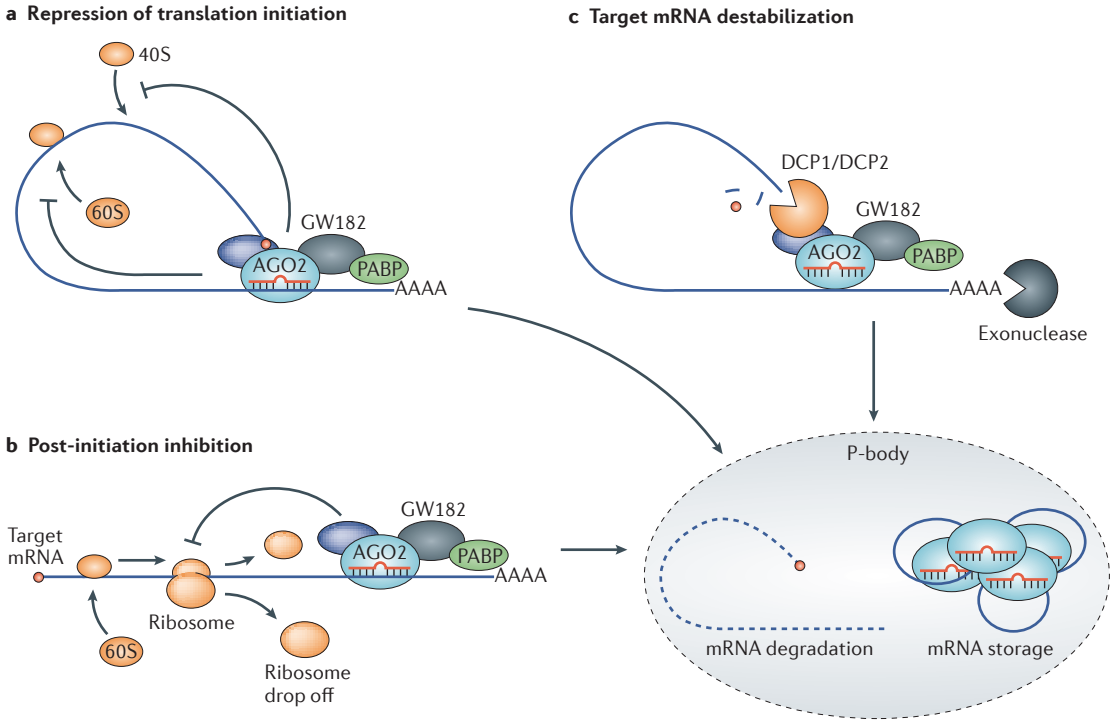

Figure 2 | **miRNA function: three potential mechanisms of miRNA-mediated post-transcriptional gene silencing.** **a** | Repression of translation initiation. MicroRNA (miRNA)-mediated silencing complexes (miRISCs) inhibit the initiation of translation by affecting eukaryotic translation initiation factor 4F (eIF4F) cap recognition, 40S small ribosomal subunit recruitment and/or by inhibiting the incorporation of the 60S subunit and the formation of the 80S ribosomal complex. Some of the target mRNAs bound by the miRISC are transported into processing bodies (P-bodies) for storage and may re-enter the translation phase when induced by exogenous signals such as stress. **b** | Post-initiation translational repression. miRISCs may inhibit the elongation of ribosomes, causing them to drop off the mRNAs and/or facilitate the degradation of newly synthesized peptides. **c** | Destabilization of target mRNAs. Binding of miRISCs to target mRNAs may recruit RNA decapping and/or deadenylating enzymes that lead to mRNA destabilization. P-bodies are the key cellular organelles for the degradation and storage of targeted mRNAs. AGO2, Argonaute 2; DCP1, mRNA-decapping enzyme 1; PABP, poly(A)-binding protein.

mRNA targets. However, unmodified RNA or DNA oligonucleotides are poorly suited to *in vivo* applications and so, in practice, chemical modification of oligonucleotides is required to increase resistance to serum nucleases, to enhance binding affinity for targeted miRNAs and to improve the PK/PD profile *in vivo*. In addition, naked oligonucleotides are incapable of penetrating negatively charged cell membranes and require modification or encapsulation to enable their entry into the cell interior. In the following sections, we review some of the chemical modifications and delivery strategies that have been developed to facilitate the therapeutic use of anti-miRs (FIG. 4). It is important to note that a substantial amount of our knowledge regarding these chemical modifications and delivery approaches was based on previous pioneering studies in the RNAi field, including siRNAs.

miRNA-targeting chemistry

The first evidence that oligonucleotides were capable of inhibiting miRNA function came from studies with unmodified antisense DNA oligonucleotides in *Drosophila melanogaster* embryos[54]. However, the sensitivity of such oligonucleotides to degradation by serum nucleases prompted the search for chemical modifications that would improve the stability and efficacy of oligonucleotides *in vitro* and *in vivo*. 2′-*O*-methyl (2′-OMe) modification of nucleotides has long been recognized to increase the resistance of oligonucleotides to nucleases and to induce rapid and stable hybridization to ssRNAs[55–58]. In 2004, two studies described the successful use of 2′-OMe-modified antisense RNAs in effectively blocking miRISCs[59,60]. In one study, a 31-nucleotide 2′-OMe-modified RNA oligonucleotide was shown to inhibit both miRISCs and siRNA RISCs (siRISCs) in *D. melanogaster* embryos and human HeLa cells. In the second study, shorter (24-nucleotide) 2′-OMe-modifed RNA oligonucleotides were able to block miRNA function *in vitro* and in cultured human cells. These studies also showed that DNA oligonucleotides had no anti-miR activity, presumably owing to degradation by endogenous DNases.

Although 2′-OMe-modified anti-miRs are more effective miRNA inhibitors than unmodified oligonucleotides are, they are still susceptible to degradation by serum exonucleases and are thus not ideal for *in vivo* applications[61]. Because exonucleases cleave the phosphate bonds

between nucleotides, modifications that block this reaction would be expected to further increase the stability of 2′-OMe-modified oligonucleotides. One strategy is to replace non-bridging oxygens in the phosphate backbone with sulphur atoms to form phosphorothioate bonds (FIG. 4). Phosphorothioate-containing oligonucleotides are less susceptible to nuclease cleavage but have reduced binding affinity for their target miRNAs; indeed, fully but solely phosphorothioate-modified anti-miRs have no miRNA-inhibitory activity[16]. Thus, selective substitution of phosphodiester bonds with phosphorothioate bonds is optimal for increasing nuclease resistance while retaining the ability to bind target miRNAs.

Phosphorothioate-modified oligonucleotides also show improved absorption, distribution and excretion profiles. It has been reported that phosphorothioate modification can enhance the binding affinity with plasma proteins, so phosphorothioate-modified oligonucleotides can be absorbed from the injection site into the bloodstream within a very short time (1–2 hours)[13,62]. Because the binding between these oligonucleotides and the tissue or cell surface is stronger than that of plasma proteins, phosphorothioate-modified oligonucleotides exhibit good uptake in several tissues, including the kidney, liver, spleen, lymph nodes, adipocytes and bone marrow, but not in skeletal muscle or the brain. Once arriving at the target organ, these oligonucleotides can be quite stable owing to the chemical modifications, and their half-life is ~1–4 weeks[62]. It seems that higher plasma protein binding (PPB) is a desirable feature for improving the PK/PD profile of ASOs. At the injection site, the concentration of oligonucleotides is very high, thus saturating binding with local tissues. Owing to their PPB, they can be transported to the bloodstream faster than other oligonucleotides with a low PPB. Once in the bloodstream, the oligonucleotides are absorbed into different tissues owing to higher binding in those regions.

The development of the next generation of anti-miRs was inspired by studies of modified siRNAs that defined the key structural and functional elements of the oligonucleotides that are required for RISC loading, target mRNA hydrolysis and catalysis. Chiu *et al.*[63,64] reported that chemical modifications were well tolerated at the 3′ end but not the 5′ end of the siRNA guide strand (antisense strand), which indicates that molecular asymmetry is important for RNAi and that the 5′ end of the antisense strand has a key role in RNAi activity. siRNAs were therefore designed with 2′-OMe and phosphorothioate modifications at the 3′ ends of both the sense and antisense strands, and linkage of cholesterol to the 3′ end of the sense strand was also found to improve the *in vivo* pharmacology and performance of the oligonucleotide[65]. siRNAs carrying these three modifications were shown to effectively silence apolipoprotein B (*Apob*) expression in the liver following intravenous injection in mice. Recent studies have demonstrated that chemically modified single-stranded siRNAs function through the RNAi pathway and potently silence mutant huntingtin protein expression in an allele-specific manner[66].

The fundamental knowledge of siRNA modifications and RISC loading prompted the adoption of similar strategies for the design of miRNA-inhibitory oligonucleotides. Krutzfeldt *et al.*[16] first reported an anti-miR oligonucleotide, antagomir-122, which carried asymmetric phosphorothioate modifications on both 5′ and 3′ ends, 2′-OMe modifications and a 3′ cholesterol tail. Antagomir-122 exhibited good efficacy and tissue distribution *in vivo* following intravenous administration in mice, although the dose was relatively high (80 mg per kg) compared with recent liposome- or conjugation-based methods that can often reach the single-digit mg per kg range. These authors also observed an antagomir-specific reduction in the expression of miR-16, -122, -192 and -194 in a range of tissues, including the liver, lung, kidney, heart, intestine, fat, skin, bone marrow, muscle, ovaries and adrenal glands[16]. The effectiveness of this approach has been confirmed by many groups and it is a more generally accepted method of miRNA inhibition[67,68].

In addition to methylation, several other modifications at the 2′ sugar position have been tested for their effect on miRNA inhibition, including 2′-*O*-methyoxyethyl (2′-MOE), 2′-fluoro (2′-F) and locked nucleic acid (LNA) modifications (FIG. 4). The 2′-MOE modification confers superior binding affinity and nuclease resistance compared with the 2′-OMe modification; indeed, the nuclease resistance of 2′-MOE-modified oligonucleotides is comparable to that of phosphorothioate-modified DNA–RNA hybrids[69]. In the first reported success with 2′-MOE-modified oligonucleotides, Esau *et al.*[12] demonstrated decreased expression of miR-143 and increased expression of putative miR-143 target genes in cultured cells. This group later reported efficient *in vivo* inhibition of miR-122 by 2′ MOE oligonucleotides; miR-122 is an miRNA that is involved in the regulation of metabolic genes that regulate cholesterol synthesis, hepatic fatty acid synthesis and oxidation in mouse hepatocytes[70]. These studies also confirmed the superior efficacy of 2′-MOE-modified oligonucleotides compared with 2′-OMe-modified oligonucleotides.

The 2′-F modification — introduction of a fluorine atom at the ribose 2′ position — differs from the 2′-MOE and 2′-OMe modifications in that it locks the sugar ring into a high 3′-endo conformation, which is often found in A-form duplexes (RNA structure) and results in exceptional affinity for target RNAs (an increase in melting temperature (T_m) of 2 °C to 3 °C per nucleotide linkage)[71]. However, 2′-F-modified oligonucleotides are not nuclease-resistant, and the phosphorothioate linkage must also be present to achieve good stability in serum. In a comparison of the effect of 2′-sugar modifications on miR-21 inhibition, Davis *et al.* showed that 2′-F-modified oligonucleotides with a phosphorothioate backbone outperformed both 2′-MOE-modified oligonucleotides with a phosphorothioate backbone and 2′-OMe-modified oligonucleotides with a phosphorothioate backbone[72]. In addition, as a recent report indicated that 2′-F-modified anti-miRs could promote protein recruitment to the anti-miR–RNA duplex[73], it is also possible that the recruitment of cellular factors could contribute in part to the superior affinity achieved by the 2′-F modification.

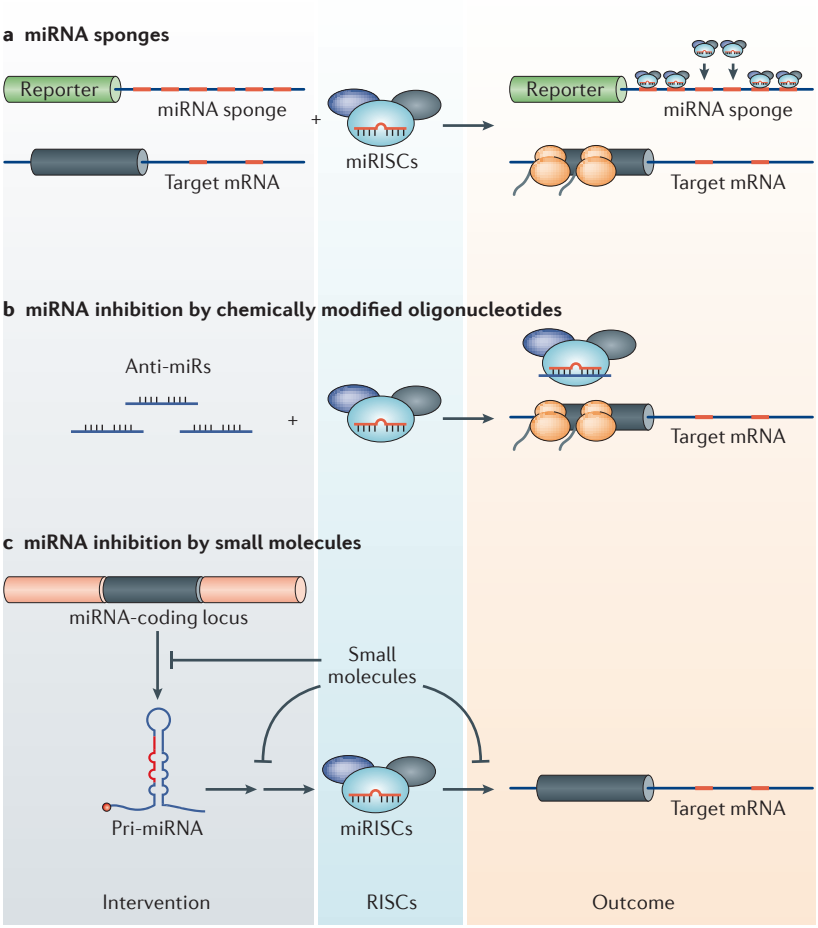

Figure 3 | **miRNA inhibition strategies.** **a** | MicroRNA (miRNA) sponges. Multiple miRNA-binding sites are inserted downstream of a reporter gene. When delivered into cells, the binding sites serve as decoys for the targeted miRNA, thereby reversing the suppression of endogenous target genes. **b** | Chemically modified miRNA-targeting antisense oligonucleotides (anti-miRs) are designed to be fully complementary to the target miRNA and bind with high affinity (high melting temperature; T_m). When delivered into cells, the anti-miRs bind to the target miRNA, relieving inhibition of the endogenous target genes. Many anti-miRs also induce degradation of targeted miRNAs. **c** | Small-molecule inhibitors can target at least three steps of miRNA assembly and function. First, small molecules can interfere with the transcription of primary miRNAs (pri-miRNAs). This inhibition could be at multiple steps, including transcription initiation, elongation and intron splicing. Second, small molecules can inhibit pri-miRNA processing by Dicer and loading into Argonaute 2 (AGO2) to form an active RNA-induced silencing complex (RISC). Third, interactions between RISC and target mRNA can be perturbed by small molecules. All of these mechanisms would lead to the loss of repression of a target mRNA by miRs. miRISC, miRNA-induced silencing complex.

LNA is another oligonucleotide modification that offers both enhanced binding affinity and good nuclease resistance. LNA is a bicyclic nucleic acid that tethers the 2′ oxygen to the 4′ carbon via a methylene bridge, locking the sugar structure into a 3′ endo conformation[74]. LNA modification offers the greatest increase in binding affinity among all the nucleic acid modifications, increasing the T_m by an average of 4 °C to 6 °C per LNA[75]. Chan *et al.*[76] first reported the use of LNA-modified anti-miRs to inhibit miRNA expression. These LNA oligonucleotides were designed with eight central LNA nucleotides flanked by seven DNA bases, and showed moderately improved efficacy compared with 2′-OMe oligonucleotides in transfected cells. Given that the flanking bases were sensitive to nuclease-mediated degradation, this result indicated that the LNA core was crucial for activity. Fully LNA-modified anti-miRs have also been analysed[77] but these showed only moderate efficiency for miRNA inhibition, possibly because of the tendency of LNA oligonucleotides to form dimers with exceptional thermal stability[78]. This problem could potentially be circumvented by reducing the number and proximity of LNAs — for example, by using a repeated pattern of two DNAs followed by one LNA. Indeed, compared with other modified anti-miRs, oligonucleotides with this design exhibited excellent miRNA-inhibitory activity at doses as low as 5 nM, and efficacy was further enhanced when the LNA substitutions were combined with other modifications, such as 2′-F[61].

Following a similar strategy, Elmen *et al.*[17,79] reported good miRNA-blocking efficacy by LNAs in mice and non-human primates, supporting the therapeutic potential of the technology. The exceptional binding affinity of LNA oligonucleotides makes it possible to achieve efficient miRNA inhibition with shorter sequences. Obad *et al.*[80] successfully used LNA-containing oligonucleotides that bound only the seed regions of the target miRNAs. This approach could potentially allow a single LNA-modified oligonucleotide to silence a family of miRNAs while avoiding the off-target effects induced by binding to the 3′ sequence of the miRNA[80]. Besides all of these classical chemical modifications on the sugar ring, emerging discoveries of non-nucleotide modifiers may provide novel insights into the development of more efficient and less toxic anti-miRs. One such example is *N*,*N*-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine (ZEN). Lennox *et al.*[81] reported that incorporation of the ZEN modification at both ends of a 2′-OMe-modified anti-miR considerably enhanced the binding affinity of such an oligonucleotide and thus resulted in more potent miRNA inhibition than its parental oligonucleotide. In addition to the increased potency and specificity, ZEN modification seems to have low toxicity in cell culture.

These examples illustrate the enormous effort made over the past decade to discover modifications that increase the binding affinity, nuclease resistance and miRNA-inhibitory activity of anti-miRs *in vitro* and *in vivo*. Strategies that combine LNA technology with other chemical modifications show particular promise for therapeutic application.

In vivo delivery strategies

Although considerable progress has been made to improve the target binding affinity and nuclease resistance of anti-miRs, there is still much work to be done in the design of vehicles for their efficient delivery *in vivo*. Most of the chemically modified anti-miR oligonucleotides show limited tissue distribution when administered in the absence of a carrier, and are taken up by the liver and kidney and rapidly excreted in urine. In addition, the dose of oligonucleotide required for *in vivo*

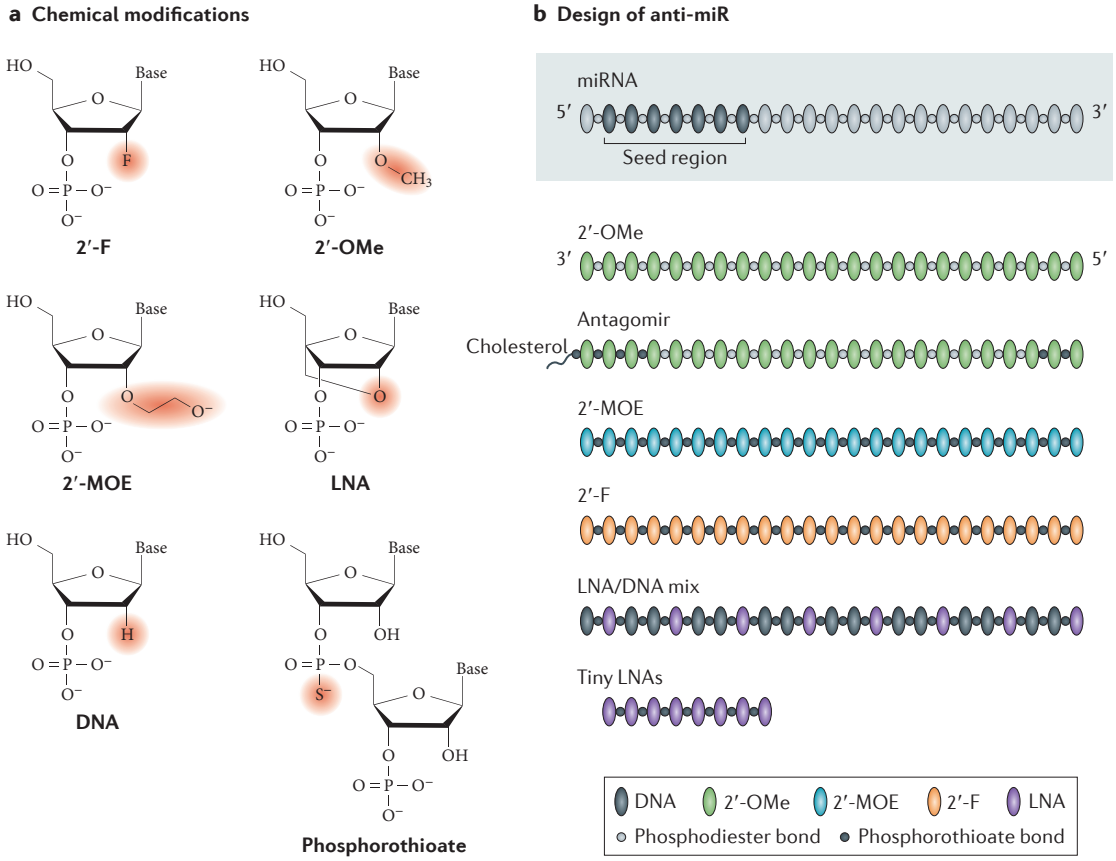

Figure 4 | **Chemically modified miRNA-targeting oligonucleotides. a** | A variety of chemical modifications have been incorporated into anti-miR oligonucleotides. Most affect the 2′ position of the sugar ring (2′-fluoro (2′-F), 2′-*O*-methyl (2′-OMe), 2′-*O*-methyoxyethyl (2′-MOE) and locked nucleic acid (LNA) modifications) and enhance the binding affinity and nuclease resistance (exo- and endonucleases) of the anti-miRs. The phosphorothioate modification is the most common change to the RNA backbone; although this further increases nuclease resistance, it decreases the microRNA (miRNA)-binding affinity of the oligonucleotide. **b** | Representative miRNA-targeting antisense oligonucleotides are shown.

inhibition is often high (~80 mg per kg for antagomirs), which increases the risk of off-target effects. Thus, an efficient *in vivo* delivery system is often needed for the therapeutic use of anti-miRs. Most of the chemically modified anti-miR oligonucleotides are negatively charged and, with the exception of the single versus double strands, have very similar properties to modified siRNAs. Thus, although many aspects of the discussion below are drawn from studies of chemically modified siRNAs, it is reasonable to assume that the same factors will influence the delivery of anti-miRs.

Conjugation-based methods. The first conjugation method reported to improve the function of anti-miRs *in vivo* was 3′ conjugation with cholesterol, which increased the inhibitory activity of the miR-122 antagomir in several tissues[16]. Later studies suggested that cholesterol-modified siRNAs that are incorporated into high-density lipoproteins (HDLs) can direct siRNAs to the liver, gut, kidney and steroidogenic organs, whereas low-density lipoprotein (LDL)-incorporated siRNAs are primarily targeted to the liver[82]. Notably, non-conjugated siRNAs did not bind appreciably to either HDL or LDL. Conjugation of α-tocopherol (a form of vitamin E) also targets siRNAs for delivery to the liver[83]. In this study, α-tocopherol was linked to the 5′ end of the antisense strand of a 27–29-mer oligonucleotide that was designed to be processed by Dicer once taken up by the cell, thus releasing the siRNA cargo. This strategy was shown to efficiently knock down *Apob* expression in mice when administered at a dose of 2 mg per kg, which is considerably lower than that required for other cholesterol-conjugated siRNAs (~50 mg per kg)[65]. Conjugation of CpG-containing oligonucleotides has been used to direct siRNAs to cells expressing Toll-like receptor 9 (TLR9), the endogenous receptor for CpG DNA. This method was used to silence the expression of the immunoregulatory transcription factor STAT3 (signal transducer and activator of transcription 3) in TLR9$^+$ myeloid cells and B cells, which enhanced an antitumour immune response and suppressed subcutaneous B16 tumour growth and metastasis *in vivo* in mice[84]. More recently, a skin-penetrating peptide was reported to successfully deliver conjugated siRNAs to keratinocytes, skin

fibroblasts and endothelial cells after topical application in mice[85]. These reports provide encouragement that selective conjugation methods can be used to efficiently deliver anti-miRs and siRNAs to target cell populations.

Liposome-based methods. Liposome-mediated delivery of siRNAs *in vivo* was first reported by Morrissey *et al.*[86], who used siRNA incorporated into a polyethylene glycol (PEG)–lipid conjugate (SNALP) to silence hepatitis B virus (HBV) replication in mice. The liposomes were efficacious at a dose of 3 mg per kg per day, which was a statistically significant improvement on the earlier study with naked siRNAs (administered at a dose of 30 mg per kg, three times a day)[87]. Mice treated with SNALP-formulated siRNAs had a tenfold reduction in serum HBV RNA compared with untreated mice[86]. siRNA–SNALPs have also been tested in monkeys, in which a single injection of 2.5 mg per kg was shown to reduce *APOB* expression by more than 90%[88]. Akinc *et al.*[89] synthesized a library of lipid-like delivery molecules (lipidoids) and showed that several exhibited excellent siRNA delivery efficiency in multiple cell lines *in vitro* as well as in mice, rats and monkeys. In addition, Akinc *et al.*[90] reported a modified liposome system that incorporated *N*-acetylgalactosamine (GalNAc)–PEG lipids to formulate siRNAs. GalNAc–PEG liposomes showed high binding affinity to asialoglycoprotein receptor (ASGPR) and resulted in enhanced siRNA delivery in the liver, achieving a remarkable ED_{50} (the median effective dose) of 0.02 mg per kg.

Peer *et al.*[91] developed a liposome-based delivery method in which siRNAs were encapsulated in 80 nm liposomes coated with hyaluronan and an integrin-specific antibody. This integrin-targeting complex effectively silenced cyclin D1 expression in leukocytes and reversed experimentally induced colitis by suppressing leukocyte proliferation and T helper 1 (T_H1) cytokine expression. Liposome-based vehicles have also been examined for localized delivery of siRNAs. Vaginal instillation of lipid-formulated siRNAs targeting herpes simplex virus 2 was able to protect mice from lethal infection for up to 9 days. The siRNAs were taken up by epithelial and lamina propria cells and did not induce the expression of interferon (IFN)-responsive genes or cause inflammation[92]. Intracranial injection of lipid-formulated siRNAs targeting conserved viral sequences protected mice from infection by the neurotropic flaviviruses, Japanese encephalitis virus and West Nile virus[93]. These reports show the promise of liposome-based delivery systems for both localized and systemic delivery of siRNA and miRNA ASOs.

Nanoparticle (polymer)-based methods. Advances in materials science and chemical engineering have led to the development of polymer-based nanoparticles as promising delivery vehicles for ASOs *in vivo*. Whereas liposomes are usually heterogeneous in size owing to interactions between water molecules and the hydrophobic groups of lipids[94], polymer-based nanoparticles with functional blocks have more flexibility on conjugations and can be produced in relatively homogeneous sizes (up to 100 nm). Several studies have shown that nanoparticle size is a critical factor for effective drug delivery *in vivo*, with particle sizes between 10 nm and 100 nm being optimal for the delivery of a variety of cargo, including small molecules, siRNAs and anti-miRs[95]. In early studies, polyethyleneimine nanoparticles were conjugated with PEG and integrin-binding RGD (Arg-Gly-Asp) peptides to form 'polyplexes' for the delivery of siRNAs into tumours[96]. However, inhibition of tumour growth was modest, probably because of the degradation of unmodified siRNA[96].

An interesting nanoparticle delivery system has been reported that can mask the immunostimulatory effects of siRNAs, even those containing known immunostimulatory sequences[97]. The particles, consisting of cyclodextrin–PEG conjugates and transferrin as the tumour-targeting ligand, successfully delivered siRNAs targeting the expression of EWS–FLI1 (a fusion protein consisting of Ewing's sarcoma breakpoint region 1 protein and Friend leukaemia virus integration 1) in a mouse model of Ewing's sarcoma[98]. The *EWS–FLI1* fusion gene is found in ~85% of patients with Ewing's family of tumours (EFTs), a devastating tumour with high metastasis and mortality rates. In non-human primates, nanoparticles containing unmodified siRNAs were well tolerated at doses between 3 mg per kg and 9 mg per kg and showed no immunostimulatory effects. A Phase I clinical trial with this delivery system provided the first evidence of RNAi-mediated target gene knockdown in patients with solid tumours[99].

Baigude *et al.*[100] reported the use of nanoparticles composed of a lysine-based amino acid backbone with lipid functional groups (iNOPs) to deliver *APOB*-targeting siRNAs *in vitro* and *in vivo*. The same system was later adapted to deliver anti-miRs, which significantly decreased miR-122 expression in the liver of mice[101]. A more recent study reported a novel nucleic-acid-based nanoparticle system that can self-assemble into particles of well-defined sizes[102]. The folate (tumour targeting)-conjugated nanoparticles improved siRNA half-life and targeted tumours with high specificity. The highly controlled and efficient assembly process for these nanoparticles suggests that they may have considerable advantages over other RNA delivery methods.

Antibody-based methods. The high affinity and binding specificity of antibodies make them attractive vehicles for cell- or tissue-specific delivery of siRNAs and anti-miRs *in vivo*. A common approach is to link an RNA-binding protein or domain to Fab fragments isolated from the cell- or tissue-targeting antibody. The first antibody-based carrier consisted of the Fab fragment of an antibody directed against the HIV-1 envelope protein gp160 fused to the nucleic acid binding protein protamine[103]. Each molecule of the fusion protein (F105-P) bound approximately six siRNA molecules and specifically delivered siRNA only to HIV Env^+ cells. In a mouse xenograft model, F105-P successfully targeted human melanoma cells expressing the HIV Env protein. This study also showed that protamine could be fused with other single-chain antibodies and that

other RNA- or DNA-binding peptides could be used for cell-type-specific delivery of siRNAs. Targeting of oligonucleotides using modified single-chain variable fragments (scFv fragments) has also been explored. In one study, a positively charged peptide containing nine arginine residues was conjugated to a carboxy-terminal cysteine residue of an scFv fragment specific for the T cell surface protein CD7 (REF. 104). After binding to CD7, the scFv–siRNA conjugate was rapidly internalized and released the siRNA in the cytosol. In a humanized mouse model of HIV infection, this system was able to knock down CC-chemokine receptor 5 (CCR5) expression and protect against HIV-induced loss of human T cells[104]. A similar study using scFv-mediated recognition of hepatitis B surface antigen and protamine as an siRNA carrier also showed significant inhibition of HBV gene expression in transgenic mice[105]. More recently, Yao *et al.*[106] also reported that the HER2–scFv protamine fusion protein can specifically deliver polo-like kinase 1 (PLK1)-targeting siRNAs into $HER2^+$ breast cancer cells in mice and resulted in significant tumour suppression. These representative studies illustrate the potential to exploit the unique binding specificity and affinity of antibodies as targeting molecules for RNAi *in vivo*.

Challenges for miRNA-targeting therapeutics

Evidence to date suggests that anti-miR-mediated silencing of miRNAs could be a powerful technology for the treatment of human disease, but it is clear that several outstanding obstacles still need to be overcome. These can be divided into three main categories: hybridization-associated and hybridization-independent off-target effects, and delivery-related issues.

Hybridization-associated off-target effects. Currently, 2,578 mature human miRNAs are registered in miRBase. From our own sequencing, as well as quantitative and biochemical analyses, only ~200 of these have sufficiently high expression to be feasible targets for mechanistic studies or therapeutic purposes. Of these, many belong to miRNA families with similar seed regions, such as the miR-17 and let-7 families. As discussed, most anti-miRs are designed to be perfectly complementary to their targets and contain chemical modifications that increase the T_m of the anti-miR–miRNA complex. Nevertheless, under physiological conditions, anti-miRs are generally unable to distinguish between miRNAs within the same family, especially those with identical seed regions. Various studies have demonstrated such promiscuous inhibition of miRNA family members by chemically modified anti-miRs. For example, a 2′-OMe-modified miR-93 inhibitor was able to inhibit other family members such as miR-106b, although a slight preference for the cognate target was observed[44]. This lack of target specificity may reflect inherent differences in traditional antisense-mediated inhibition of mRNAs and miRNAs. For mRNA silencing, chemically modified ASOs are designed to bind to the protein-free, full-length target mRNA rather than to a functional RNA–protein complex. By contrast, mature miRNAs within RISCs are always bound by Argonaute proteins to form functional RISCs; therefore, the binding between ASOs with target miRNAs follows the same rule as that between RISCs and their downstream target mRNAs.

As mentioned earlier, the importance of the miRNA seed region for miRNA targeting was revealed by Obad *et al.*[80], who showed that LNA-containing anti-miRs targeting the seed regions effectively blocked the expression of miRNAs from the same family, whereas short LNAs targeting the 3′ sequence had no inhibitory effect, which indicates that the latter sequence has no role in gene silencing. Conversely, there have been reports of successful targeting of the 3′ sequence of miRNAs[107], which suggests that further work is necessary to understand the importance of this region for anti-miR targeting. Thus, the ability of individual anti-miRs to cross-inhibit molecules with a common seed region highlights the need for caution during the development of therapeutics targeting a single miRNA.

Although there is limited space for potentiating the targeting specificity of anti-miRs for single miRNAs owing to the conservation of seed regions, an alternative strategy in which miRNAs are targeted at their precursor stage may become a valid approach for addressing this issue. Kloosterman *et al.*[108] reported that the miRNA biogenesis and maturation process could be efficiently inhibited by morpholinos in an miRNA-specific manner. It was shown that inhibition of miR-375 would lead to defective morphology of pancreatic islet cells, and this phenotype could be observed with multiple precursor-targeting morpholinos. Although these experiments were carried out in zebrafish embryos, morpholinos and other chemically modified oligonucleotides have been tested for binding to their target mRNA and inhibiting its splicing or translation in mammals, and some have been tested in clinical trials as well. For instance, drisapersen (developed by Prosensa Therapeutics) is a 2′OMe-modified full phosphorothioate ASO that can bind to an exon-internal site of dystrophin pre-mRNA and induce exon skipping during splicing, which has been shown to improve muscle function in patients with Duchenne muscular dystrophy (DMD)[109,110].

Therefore, as miRNA biogenesis is a multi-step process and requires strong secondary structures to recruit the enzymes that are involved, a similar strategy can be used to target miRNA expression by disrupting the generation of its precursor. This strategy may also help to overcome the limitation caused by targeting mature miRNAs, where only inhibition of the seed region matters and there is very limited flexibility for targeting individual miRNAs from the same family. As pri-miRNAs usually contain sequences not found in mature miRNAs, and those sequences are not conserved among different miRNAs (even from the same family), chemically modified short oligonucleotides can thus be designed to bind specifically to these sequences. As these oligonucleotides have high binding affinity, it is quite feasible that they can disrupt the hairpin structure of the targeted miRNA and cause defects in its further processing by the Drosha–DGCR8 complex, thus reducing the level of downstream mature miRNA. Meanwhile, the specificity of this approach can be validated using independent

oligonucleotides that target different parts of the miRNA precursors, thus helping to exclude any oligonucleotide-specific off-target effects. However, there is surprisingly limited information available in the literature on such a strategy; moreover, most — if not all — miRNA-targeting approaches were focused on targeting their mature forms. Thus, further efforts are warranted to explore whether targeting miRNA at the precursor level may be a promising approach for addressing the specificity issue caused by cross-family miRNA inhibition.

Hybridization-independent off-target effects. Anti-miRs and carrier proteins may be detected by both the innate (nucleotide sequence) and adaptive (carrier and/or nucleotide) arms of the mammalian immune system. Indeed, immunostimulatory off-target effects are serious toxicological concerns for oligonucleotide therapeutics. Cells of the innate immune system express TLRs, an ancient family of pattern recognition receptors that have an essential role in microbial defence[111]. Among these are TLR3, TLR7, TLR8 and TLR9, which are endolysosomal receptors that recognize RNA (TLR3, TLR7 and TLR8) and DNA (TLR9) of bacterial and viral origin[111]. TLR3 recognizes dsRNA ligands and is activated by siRNAs in a sequence-independent manner[112,113], whereas TLR7 and TLR8 predominantly bind ssRNAs. Early studies suggested that TLR7 and TLR8 preferentially recognized GU-rich RNA sequences[114,115], and siRNAs lacking GU-rich motifs were shown to have reduced immunostimulatory effects[116]. However, such siRNAs were still able to evoke an IFNα response by plasmacytoid dendritic cells[117], which, according to a more recent report, is possibly due to the presence of a non-U-rich motif that can stimulate IFNα response in a TLR7-dependent manner[118].

Hornung *et al.*[117] further proved that immune activation is mediated by the siRNA sense strand, which is equivalent to the anti-miR antisense strand that is fully complementary to the RISC-bound miRNA. Replacement of sense strand nucleotides with poly(A), which is not immunostimulatory, led to the identification of nine nucleotides in the 3′ end of the sense strand as being responsible for TLR3 activation. Interestingly, this motif covers the entire seed region of the RISC-bound antisense strand. Notably, LNA modification of the 2′ position of the sugar ring largely reduced the immunostimulatory effects of siRNAs. This study also demonstrated that the minimal ssRNA length required to induce IFNα expression was ~12 nucleotides. These data provide valuable insight for the design of anti-miR oligonucleotides, substantiate the efficacy of short sequences that target only the miRNA seed region, and further demonstrate that the immunostimulatory effects of siRNAs can be minimized by chemical modification at the 2′ position of the sugar.

Although chemical modification of oligonucleotides is necessary to increase their RNA-binding affinity and nuclease resistance, these changes are known to induce sequence-independent toxicity *in vivo*[119]. The most commonly observed effects are inhibition of coagulation, activation of the complement cascade and immune cell activation[119]. Phosphorothioate-containing oligonucleotides, for example, inhibit coagulation and transiently prolong clotting time in monkeys[120]. This effect was sequence-independent but correlated with plasma concentrations of the oligonucleotide[120]. Phosphorothioate-modified oligonucleotides can also cause complement-mediated toxicity. In monkeys, phosphorothioate-modified ASOs, ranging from 20 to 33 nucleotides in length, were shown to activate C5 complement and induce a transient decrease in peripheral white blood cell counts[121]. Similar effects have been reported for anti-miRs[122]. For example, miravirsen, an LNA-modified anti-miR-122 oligonucleotide that is currently in clinical trials for HCV infection, was found to transiently prolong clotting time, modestly activate the alternative complement pathway and reversibly increase hepatic transaminases in monkeys. Although these effects were observed at high doses, and most ASOs are well tolerated[119], the data do highlight the potential toxicity of anti-miRs. Thus, continued efforts to improve the PK/PD profile of oligonucleotides and develop more efficient delivery systems will be needed to increase the therapeutic window and avoid effects on coagulation, complement activation and immunostimulation.

Liver toxicity is another outstanding concern of chemically modified ASOs. One such example is LNA-modified oligonucleotides. Swayze *et al.*[123] reported that although some LNA-modified oligonucleotides exhibited higher potency than traditional 2′-MOE-modified ones, they also had profound hepatotoxicity issues as measured by serum transaminase activity as well as organ and body weight during preclinical animal tests. The hepatotoxicity seemed to be induced in a sequence-independent manner, as multiple LNAs targeting different molecules — as well as mismatched non-targeting control oligonucleotides — showed similar toxicity issues. In a recent report, Stanton *et al.*[124] evaluated the toxicological impact of subtle changes in the chemical modification patterns of ASOs. The key finding in their report indicated that even for the same oligonucleotide, subtle changes in the position or pattern of the modification could result in substantial changes in the severity of the induced hepatotoxicity. Similar findings were also reported by Kakiuchi-Kiyota *et al.*[125]. It was noted that different hepatotoxic LNAs seemed to affect distinct mechanistic pathways, which suggests that the toxicity issues associated with ASOs may vary dramatically with different sequences. These observations highlight the need for careful evaluation of chemical modification combinations to develop a robust but less toxic ASO candidate, and this should be addressed in a case-by-case manner.

Delivery-related concerns. Hundreds of miRNAs have been reported to be involved in disease development and progression, especially in tumours. Owing to their targeting of multiple genes, often within the same pathway, miRNAs that exhibit aberrant expression in diseased tissues are attractive therapeutic candidates as their inhibition could have systemic effects. Depending on the disease and target tissues, different strategies will

need to be carefully considered in order to achieve the desired delivery of anti-miRs. For example, some tissues such as the liver, kidney and spleen — and, to a certain extent, lung — are known to be more accessible than others. For these tissues, it is fairly easy to achieve a sufficient delivery dose by using ASOs alone without carriers[62]. However, carefully designed and formulated carrier particles have to be included to help ASOs reach hard-to-reach tissues such as solid tumours. As these delivery carriers are far from perfect in delivering ASOs to target tissues, on-target side effects may result from interference with miRNAs expressed in normal tissues. One such example is the miR-17–92 cluster of miRNAs, which is highly induced in many solid tumours and haematological malignancies[126]. Overexpression of miR-17–92 enhances cell proliferation and reduces apoptosis, probably through its effects on BCL-2-interacting mediator of cell death (*BIM*), phosphatase and tensin homolog (*PTEN*) and p21 (*CDKN1A*) gene expression[126].

At face value, targeting such 'oncogenic' miRNAs seems to be a reasonable approach for inhibiting tumour growth and, consistent with this, anti-miR oligonucleotides have been shown to selectively induce apoptosis and growth arrest in human lung cancer cell lines overexpressing miR-17–92 (REF. 127). However, this miRNA cluster is expressed in many normal tissues, and targeted deletion of miR-17–92 in mice causes death of neonates owing to lung hypoplasia and a ventricular septal defect[128]. miR-17–92 is also required for normal development of B cells[128] and follicular T helper cells[129], revealing an important regulatory role in immune homeostasis. These findings suggest that anti-miR-mediated targeting of the miR-17–92 cluster could not only induce developmental defects but also compromise immunity and increase the risk of infection. This example illustrates the need to develop targeted delivery approaches that minimize potentially deleterious effects in normal tissues. One such strategy that has received increasing attention over the years is to utilize the specific antigens expressed on the tumour cell surface. By using ligands, peptides or antibodies — which can specifically recognize and interact with these antigens — to coat the surface area of formulated anti-miR-containing nanoparticles, anti-miRs can be more efficiently delivered into tumour tissues and achieve efficient miRNA inhibition.

There are several aspects to be carefully considered when developing such a targeted delivery system. First, anti-miRs are generally negatively charged, so the nanoparticle block should contain a positively charged domain to efficiently bind the anti-miRs. Second, the nanoparticle complex should be coated with targeting molecules that can specifically interact with the target antigen on the cell surface. These targeting molecules could help retain the anti-miR-containing complex in the target tissue and ensure that it binds with target cells. The outer surface of the nanoparticle should also contain hydrophilic groups in order to avoid rapid clearance by the reticuloendothelial system and enhance circulation time when injected into the bloodstream[130]. Last, the size of the final complex should be less than 100 nm in diameter. Ideally, the formulated anti-miR-containing complex should have a uniform (or narrow) size distribution and be big enough to avoid fast clearance through the kidney excretion system but small enough to penetrate to the target tissue[130,131].

Another delivery-related concern associated with the therapeutic use of ASOs is how to ensure that an effective dose reaches the appropriate target cells. In the case of siRNAs and miRNA inhibitors, this requires an understanding of the proportion of the administered oligonucleotide that is delivered to the tissues, released within cells and becomes incorporated into RISCs. For efficient delivery of ASOs into the target tissue, knowledge of the tissue architecture and the context of the local microenvironment is necessary. For example, solid tumours exhibit considerable heterogeneity in their architecture. In general, four regions can be recognized in these tumours based on perfusion rates, including a necrotic centre, a seminecrotic intermediate region, a peripheral highly vascularized region and an advancing front[132]. Owing to the rapid angiogenesis process, tumour vessels exhibit several abnormalities compared with normal blood vessels, including high permeability and hydraulic conductivity[132]. As these leaky vessels also have a finite pore size, ASO-containing tumour-targeting nanoparticles have to reach an appropriate size distribution in order to penetrate the tumour tissues more efficiently. Once the ASO-containing complex reaches tumour tissues, another obstacle it faces is the high interstitial pressure that slows down the diffusion and convection of nanoparticles within the tissue. The surface antigen-binding groups coated on those nanoparticles are then important for promoting the retention and internalization of cargos.

Moreover, even when anti-miRs are delivered into target tissues at therapeutically meaningful doses, another limiting factor is determining how to reach a sufficient dose within the cells in order to achieve efficient miRNA inhibition. Notably, the efficiency of the internalization and release of anti-miRs is extremely low. Gilleron *et al.*[133] recently reported an imaging-based analytical method using fluorescence and electron microscopy to track the intracellular transport and release of siRNAs. They showed that lipid nanoparticles (LNPs) entered the cells through clathrin-mediated endocytosis and macropinocytosis, and only 1–2% of the payload escaped from endosomes into the cytosol[133]. These data indicate that a delivery system designed to facilitate the release of oligonucleotides from endosomes would considerably decrease the therapeutic dose of siRNAs or miRNA-targeting ASOs *in vivo*.

Clinical development of miRNA therapeutics

miRNAs and miRNA-targeting oligonucleotides have several advantages over traditional small-molecule drugs, most notably the ease with which oligonucleotides can be chemically modified to enhance their PK/PD profiles and the ability of miRNAs to target multiple genes simultaneously. Not surprisingly, miRNA-targeting therapies are an area of intense interest to pharmaceutical companies, and many such compounds are in preclinical and clinical development for a variety of indications (TABLES 1,2).

Table 1 | **Selected anti-miR therapeutics currently in development**

MicroRNA	Oligonucleotide format	Indications	Companies	Developmental stage
miR-122	LNA-modified antisense inhibitor	HCV infection	Santaris Pharma	Phase II
miR-122	GalNAc-conjugated antisense inhibitor	HCV infection	Regulus Therapeutics	Phase I
miR-34	miRNA mimic replacement	Liver cancer or metastasized cancer involving liver	miRNA Therapeutics	Phase I
Let-7	miRNA mimic replacement	Cancer (details undisclosed)	miRNA Therapeutics	Preclinical
miR-21	2′-F and 2′-MOE bicyclic sugar modified antisense inhibitor	Cancer, fibrosis	Regulus Therapeutics	Preclinical
miR-208	Antisense inhibitor	Heart failure, cardiometabolic disease	miRagen/Servier	Preclinical
miR-195 (miR-15 family)	Antisense inhibitor	Post-myocardial infarction remodelling	miRagen/Servier	Preclinical
miR-221	Antisense inhibitor	Hepatocellular carcinoma	Regulus Therapeutics	Preclinical
miR-103/105	Antisense inhibitor	Insulin resistance	Regulus Therapeutics	Preclinical
miR-10b	Antisense inhibitor	Glioblastoma	Regulus Therapeutics	Preclinical

2′-F, 2′-fluoro; 2′-MOE, 2′-*O*-methyoxyethyl; GalNAc, *N*-acetylgalactosamine; HCV, hepatitis C virus; LNA, locked nucleic acid; miRNA, microRNA.

Although this Review focuses on targeting miRNAs by anti-miRs, we include the clinical development of miRNA replacement therapies in this section in order to provide a clearer image on the progress of the field.

miR-122 and HCV infection. miR-122 was identified in 2005 as a liver-specific miRNA that can modulate HCV replication. Consistent with this, HCV replication was shown to be inhibited by a 2′-OMe-modified anti-miR-122 ASO, paving the way for the development of miR-122 inhibitors as treatments for HCV infection in humans[134]. One of these, miravirsen, is currently in clinical trials and is the leading prospect for antisense therapy of HCV infection. Miravirsen is a 15-mer LNA- and phosphorothioate-modified anti-miR with a high affinity for miR-122 (with a T_m of 80 °C). Preclinical studies in healthy monkeys confirmed that miravirsen effectively suppressed miR-122 expression and had a favourable toxicity profile[17,122], and in chimpanzees with chronic HCV infection a dose of 5 mg per kg decreased HCV infection by two orders of magnitude[15]. These encouraging data were followed by clinical studies. Miravirsen was well tolerated and displayed no dose-limiting toxicity in Phase I single-dose (12 mg per kg) and multiple ascending-dose (up to five doses of 1–5 mg per kg) studies in healthy individuals[135]. Although several small-molecule-based therapeutic agents targeting proteins encoded by the HCV genome have shown clinical efficacy in Phase III trials, molecules such as miR-122 that target host proteins could be used to overcome the resistance mutations that arise in HCV.

In a Phase II study, patients with chronic HCV infection received five weekly subcutaneous injections of miravirsen, which resulted in a mean 2–3 log decrease in serum HCV RNA, and HCV RNA was undetectable in four of the nine patients who received the highest dose tested (7 mg per kg). No serious adverse effects were reported[135]. These impressive data suggest that miravirsen may become the first anti-miR oligonucleotide drug to enter the market.

miR-34a, miR-34b, miR-34c and cancer. The miR-34 family of miRNAs (composed of miR-34a, miR-34b and miR-34c) was first associated with human cancer in 2004, when the coding locus was linked with genomic regions that are frequently mutated in cancer[8]. Since then, many reports have shown that miR-34 is a crucial regulator of cell growth in various types of cancer, including liver and lung cancers[136]. miR-34 inhibits cell growth by targeting a group of oncogenes involved in cell cycle control (cyclin-dependent kinase 4 (*CDK4*), *CDK6* and the transcription factor *E2F3*), proliferation (*MYC* and histone deacetylase 1 (*HDAC1*)), apoptosis (B cell lymphoma 2 (*BCL-2*) and sirtuin 1 (*SIRT1*)) and metastasis (*WNT* and metastasis-associated protein *MTA2*). Perhaps not surprisingly, miR-34 expression is reduced in many cancer cells[136]. These data suggested that miR-34 mimics might be promising therapeutic options for reinstating the normal regulation of a range of cell death and survival genes in cancer cells. The leading therapeutic, MRX34, is a lipid-formulated miR-34 mimic under development by Mirna Therapeutics[137] that

Table 2 | **RNAi therapeutics currently in development**

Targets	Drug	Indications	Developers	Developmental stage
CTNNB (encodes β-catenin)	CEQ508	Familial adenomatous polyposis	Marina Biotech	Phase II
N gene of RSV	ALN-RSV01	Respiratory syncytial virus	Alnylam Pharma	Phase II
TTR	ALN-TTR02	TTR-mediated amyloidosis	Alnylam Pharma	Phase II
TP53	QPI-1002	Acute kidney injury; delayed graft function	Quark	Phase II
KSP, *VEGF*	ALN-VSP	Liver cancers	Alnylam Pharma	Phase I
DDIT4	PF-04523655	Age-related macular degeneration)	Pfizer/Quark	Phase II
FURIN	FANG vaccine	Solid tumours	Gradalis	Phase II
PCSK9	ALN-PCS	Hypercholesterolaemia	Alnylam Pharma	Phase I
PLK1	TKM-PLK1	Advanced solid tumours	Tekmira	Phase II
CTGF	RXI-109	Scar prevention	RXi Pharma	Phase II
CASP2	QPI-1007	Ocular neuroprotection; non-arteritic anterior ischaemic optic neuropathy	Quark	Phase I
STMN1	pbi-shRNA STMN1 lipoplex	Advanced and/or metastatic cancer	Gradalis	Phase I
Not disclosed	ARC-520	Hepatitis B virus infection	Arrowhead Research Corporation	Phase I
PCSK9	SPC5001	Hypercholesterolaemia	Santaris Pharma	Phase I
APOB	SPC4955	Hypercholesterolaemia	Santaris Pharma	Phase I
PSMB8, *PSMB9* and *PSMB10*	NCT00672542	Metastatic melanoma vaccine	Duke University	Phase I
PKN3	Atu027	Solid tumours	Silence Therapeutics	Phase I
Ebola virus	TKM-Ebola	Zaire species of Ebola virus	Tekmira	Phase I

APOB, apolipoprotein B; CASP2, caspase 2; CTGF, connective tissue growth factor; DDIT4, DNA-damage-inducible transcript 4; KSP, kinesin spindle protein; PCSK9, proprotein convertase subtilisin kexin 9; PKN3, protein kinase N3; PLK1, polo-like kinase 1; PSMB, proteasome subunit beta type; RNAi, RNA interference; RSV, respiratory syncytial virus; STMN1, stathmin 1; TP53, tumour suppressor p53; TTR, transthyretin; VEGF, vascular endothelial growth factor.

inhibits tumour growth and increases overall survival in mouse models. MRX34 is the first miRNA mimic to enter clinical trials and is currently in Phase I testing in patients with primary liver cancer or metastatic cancer that has spread to the liver.

Let-7 and cancer. The miRNA let-7 is one of the earliest discovered miRNA genes that can regulate developmental processes in *C. elegans*[138]. The close association of let-7 expression with cancer was first discovered by Takamizawa *et al.*[139] who showed that reduced let-7 expression correlated with significantly shorter survival in patients with lung cancer. This correlation was later proposed to be due to the let-7-mediated inhibition of *RAS*, which is a critical oncogene that is involved in lung cancer development[140]. Reduced let-7 was also found to lead to increased expression of high-mobility group AT-hook protein 2 (HMGA2), which enhanced anchorage-independent cell growth and tumour transformation[141]. Moreover, two independent groups reported the *in vivo* tumour-suppressive role of let-7 (REFS 142,143). Let-7 was shown to induce growth arrest in multiple cancer cell lines, especially those with *KRAS* mutations, and to suppress tumour growth in a xenograft model of human lung cancer[142,143]. In addition to lung cancer, let-7 was found to suppress the growth of other cancer cells, including breast cancer cells[144]. These data suggest that the delivery of let-7 miRNA into tumours may have therapeutic benefit in patients with cancer. Mirna Thearpeutics is currently developing let-7 as a potential miRNA replacement treatment for cancer. Although details of the cancer type have not been disclosed, it will be interesting to see whether such miRNA mimetic delivery could indeed have a therapeutic impact.

miR-21 and cancer. The link between miR-21 and cancer was first discovered by Volinia *et al.*[145], who found that miR-21 is overexpressed in the majority of tumour samples. This observation was later confirmed in many types of cancer, including both solid tumours and haematopoietic cancers[146]. Mechanistically, overexpression of miR-21 leads to the suppression of several key tumour suppressor genes, such as *PTEN*[147], tropomyosin 1 (*TPM1*)[148]

and programmed cell death protein 4 (*PDCD4*)[149]. Thus, miR-21 was identified as one of the oncomiRs whose inhibition may have therapeutic benefits. Indeed, in glioblastoma cells, inhibition of miR-21 was found to promote cancer cell death[76], which was also confirmed in liver and breast cancer cells[147,150]. Therefore, miR-21 inhibition was chosen as one of the promising therapeutic strategies for treating hepatocellular carcinoma, and a miR-21 inhibitor is currently being developed by Regulus Therapeutics. Meanwhile, it was also reported that miR-21 upregulation promoted fibrosis in both the heart and kidney in animal models[67,151], which indicates that its inhibition may be a promising antifibrotic therapeutic approach as well.

miR-208 and cardiac diseases. miR-208 is one of the specific miRNAs that is highly expressed in the heart[152]. It is encoded in the intron region of the human and mouse αMHC (α-myosin heavy chain) genes[152]. Van Rooij *et al.*[152] reported that miR-208-knockout mice developed normally. However, these mice experienced gradual loss of cardiac function and failed to initiate cardiac hypertrophic growth in response to pressure-overload stress[152]. Van Rooij *et al.* further proposed that the phenotype was likely to be due to miR-208-mediated regulation of the expression of thyroid hormone receptor-associated protein 1 (THRAP1; also known as MED13), which is a key component of the thyroid hormone signalling pathway[152]. More recently, the therapeutic effect of inhibiting miR-208 was first described by Montgomery *et al.*[153], who showed that miR-208 inhibition by an LNA-modified anti-miR could protect rats from hypertension-induced heart failure. However, owing to the high dose needed (25–33 mg per kg) to achieve sufficient miR-208 inhibition and the gradual loss of cardiac function in miR-208-null animals, there is a high risk of cardiac toxicity associated with the potential development of such a therapy. The therapy is being developed by miRagen Therapeutics and is currently at the preclinical stage.

miR-15, miR-195 and heart regeneration. The miR-15 family miRNA miR-195 was highly induced in cardiac ventricles during the postnatal switch to the terminally differentiated stage, when neonatal cardiomyocytes begin to withdraw from the proliferation stage of the cell cycle[154]. Overexpression of miR-195 in the embryonic heart led to ventricular hypoplasia and septal defects[154]. A mechanistic study revealed that miR-195 regulated cardiomyocyte proliferation by targeting a number of cell cycle genes, including checkpoint kinase 1 (*CHEK1*)[154]. Notably, inhibition of miR-195 by LNA-modified anti-miRs in mice and pigs showed strong effects on cardiac regeneration, and treated animals were protected from myocardial infarction — the most common antecedent of heart failure in humans[155,156]. Currently, an agent targeting miR-15/195 is being co-developed by miRagen Therapeutics and Servier, and is at the preclinical stage.

miR-221 and hepatocellular carcinoma. miR-221 was found to be upregulated in many cases of human hepatocellular carcinoma (HCC)[157]. Mechanistic studies indicated that miR-221 can target p57 (also known as CDKN1C), p27 (also known as CDKN1B) and BCL-2-modifying factor (BMF) expression in HCC cells[157,158], and overexpression of miR-221 stimulated the growth of tumorigenic murine hepatic progenitor cells in a mouse model of liver cancer[159]. Blocking of miR-221 by chemically modified anti-miRs led to decreased tumour growth and increased survival in animal models[160]. A miR-221-blocking anti-miR is currently being developed by Regulus Therapeutics in partnership with Sanofi, and is at the preclinical stage.

miR-103, miR-107 and insulin sensitivity. miR-103 and miR-107 are located within the intronic regions of pantothenic acid kinases (PANKs) and their expression was upregulated in leptin-deficient (*ob/ob*) and diet-induced obese (DIO) mice[161]. In mouse models, overexpression of these miRNAs caused dysregulated glucose homeostasis, whereas anti-miR-mediated inhibition improved insulin sensitivity and glucose homeostasis[161]. Mechanistic studies revealed that miR-103 and miR-107 could function by directly targeting the voltage-gated calcium channel Cav1, which is a critical regulator of the insulin receptor. Thus, miR-103 and miR-107 could be promising targets for obesity-related insulin resistance. Currently, an anti-miR is being developed by Regulus Therapeutics in partnership with AstraZeneca, and is at the preclinical stage.

Many additional miRNA mimics, anti-miR oligonucleotides and siRNA therapeutics are in development (TABLES 1,2). Most of the miRNA-targeting molecules are still at the preclinical stage but have shown efficacy in various animal models of disease. Given the advances over the past decade in oligonucleotide chemistry and delivery technologies, we are optimistic that many miRNA therapies will follow the examples of miR-122 anti-miRs and miR-34 mimetics to form a novel class of drugs for the treatment of various diseases.

Future perspectives

An important question that remains to be answered is what the potential drug resistance mechanisms are for miRNA-inhibiting oligonucleotide therapeutics. There could be three potential mechanisms. The first is a change in the ADME (absorption, distribution, metabolism and excretion) of anti-miRs. It could include upregulation of particular pumps, metabolic enzymes that can help to remove the drugs from the cells and enhance its degradation through enzymatic activities, as well as the development of neutralizing antibodies that can help with drug clearance. The second mechanism involves upregulating the expression of targeted miRNAs or enhancing their biogenesis and processing to counteract the effect of miRNA inhibition. The third mechanism involves upregulating other miRNAs that can target the same genes, thereby counteracting the effect of miRNA inhibition. Investigating and understanding the mechanisms of drug resistance could promote further optimization of miRNA-targeting strategies and lead to the development of next-generation therapies.

As the field continues to evolve, a better and more thorough understanding of miRNA biogenesis and function will also help to guide future endeavours in miRNA

therapeutics. Current knowledge of miRNA function has largely focused on post-transcriptional gene silencing induced by the binding of miRISC to the 3′ untranslated region of the targeted mRNA. However, this represents only one aspect of miRNA function and we will undoubtedly discover other miRNA-modulated biological processes that could serve as novel therapeutic targets. Indeed, recent evidence suggests that miRNAs may have a range of functions, including regulation of transcription through epigenetic mechanisms[162], regulation of translation by acting as a decoy[163] and regulation of other long non-coding RNAs[164–166]. Given that post-transcriptional modifications (for example, methylation)[167] have important roles in regulating the stability and translation of mRNAs, it will also be interesting to determine whether miRNAs might be involved in this aspect of mRNA regulation.

Considerable work will be necessary to develop more efficient vehicles for the targeted delivery of oligonucleotides to specific organs, tissues and cell types. To date, all forms of miRNA-targeting oligonucleotides, including liposome-encapsulated, nanoparticle-associated and naked oligonucleotides, have been found to localize primarily to the liver, spleen and kidney. At present, oligonucleotides can only be administered through the intravenous or subcutaneous routes, and the development of oral delivery vehicles will clearly be an important step in advancing this class of drugs through clinical development to routine use in patients.

Finally, it will be interesting to determine whether miRNA-targeting therapeutics could be combined with other chemical or biological drugs for multidrug therapy. Many human diseases are driven by multiple cellular pathways that act in concert; for these conditions the inhibition of a single target may have limited efficacy and, in some cases, be actively deleterious. For example, many therapy-resistant cancers display a more aggressive disease evolution with poor prognosis. By targeting multiple pathways simultaneously, combinatorial treatments could reduce the risk of such resistance emerging. This approach will require a greater understanding of how drug treatment influences miRNA expression and function to ensure that the most appropriate miRNAs are targeted.

Despite the outstanding obstacles, we have clearly arrived at a point where the targeting of miRNA function by mimics or inhibitors has become a viable option for the modulation of many aspects of human disease. We are optimistic that an increasing number of these molecules will progress through clinical development and become approved treatments in the coming years.

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Acknowledgements
We are grateful to our colleague Dr R. Zhou and members of the Rana laboratory for helpful discussions. We apologize to our colleagues whose work we could not cite owing to space limitations. This work was supported in part by grants from the US National Institutes of Health (to T.M.R).

Competing interests statement
The authors declare no competing interests.

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