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Review

Emerging concepts of miRNA therapeutics: from cells to clinic

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MicroRNAs (miRNAs) are very powerful genetic regulators, as evidenced by the fact that a single miRNA can direct entire cellular pathways via interacting with a broad spectrum of target genes. This property renders miRNAs as highly interesting therapeutic tools to restore cell functions that are altered as part of a disease phenotype. However, this strength of miRNAs is also a weakness because their cellular effects are so numerous that off-target effects can hardly be avoided. In this review, we point out the main challenges and the strategies to specifically address the problems that need to be surmounted in the push toward a therapeutic application of miRNAs. Particular emphasis is given to approaches that have already found their way into clinical studies.

Highlights

Single microRNAs (miRNAs) regulate large subsets of mRNA targets. Although this property makes miRNAs potentially a powerful therapeutic tool, it also represents a major challenge in terms of controlling adverse effects that have been observed in clinical trials.

Besides systemic applications via injection and infusion, advanced strategies emerge for miRNA-based drug administration via implantable 3D matrices, inhalation schemes, and intake via food.

A combination of miRNA therapeutics with chemical modifications, biomolecule conjugation, or the use of carriers improves a site-directed and efficient cell targeting.

A comprehensive risk assessment of miRNA therapeutics is required before any *in vivo* targeting to minimize off-target effects and to avoid overdosing of miRNAs.

What are the promises of miRNA therapeutics?

miRNAs (see Glossary) are small, noncoding RNAs that serve as post-transcriptional regulators of protein encoding genes. There are more than 2300 different miRNAs in human cells with time- and tissue-dependent expression patterns [1–3]. Essential aspects of miRNA biogenesis and its functionality are provided in Box 1. Criteria of miRNA fidelity are addressed below.

Physiological changes of miRNA expression are pivotal to regulate complex genetic networks and in consequence cellular signaling cascades. In many disease scenarios, altered miRNA expression plays likewise a central role in modifying the protein expression as part of pathological cellular changes [4]. Besides the diagnostic potential of altered miRNA expression levels, these small RNAs offer themselves for therapeutic purposes toward a targeted manipulation of cell functions that are crucial to a disease phenotype [5]. What makes an miRNA-based intervention most efficient, and consequently especially attractive, is the broad spectrum of targets that can be regulated by a single miRNA [6]. Thus, a single miRNA can direct entire cellular pathways in spite of a relatively moderate effect on each of the targeted genes, as shown for miR-34a-5p that has been identified as a hub of T cell regulation networks [6,7]. Vice versa, one gene or one pathway is typically regulated by several miRNAs, resulting in a complex and powerful regulatory network, potentially addressing the majority of molecular pathomechanisms in humans.

Against this background, it is not surprising that, according to PubMed records, since 2015, more than 600 articles have been published under the heading of 'miRNA-based therapeutics'. Although a future therapeutic use of miRNAs is undoubtedly appealing, there are still great practical difficulties to overcome, including the identification of proper administration routes, the control of in-body stability, the targeting of specific tissues and cell types, and the attaining of the intended intracellular effects. Hence, only few miRNA-based drugs have, as of now, entered a clinical test phase (Table 1). In the following sections, we address the different challenges on the way to an effective and nonhazardous use of miRNA therapeutics. We particularly emphasize preclinical studies that developed strategies to address specific challenges associated with using miRNA therapeutics.

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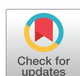

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Box 1. Cellular miRNA biogenesis

miRNA encoding sequences are located in exons or introns of protein-encoding genes or in intergenic regions. They can be coregulated together with their host genes or can be under the control of their own promoters [103]. During their biogenesis (thoroughly reviewed elsewhere, e.g., [103–105]), the miRNA encoding sequences are transcribed by RNA polymerase II or III to form primary miRNAs (pri-miRNAs), which are up to several thousand nucleotides in length and shaped as a hairpin structure. pri-miRNAs are further processed by the Drosha-DGCR8 microprocessor complex in the nucleus to generate an miRNA precursor (pre-miRNA) of approximately 70 nucleotides in length. In a noncanonical biogenesis, intron-encoded pre-miRNAs (mirtrons) can be directly processed along with their coencoded transcripts through spliceosomes. The pre-miRNA hairpin is exported by exportin-5 to the cytoplasm, where it is cleaved into an miRNA duplex of approximately 22 nucleotides in length by the RNase Dicer and the double-stranded RNA binding enzyme TRBP. Single miRNA strands are subsequently incorporated into the RISC, allowing the ribonucleoprotein complex to bind to target sequences that are usually located within the 3′ untranslated regions of the mRNAs. Reverse complementary binding takes place in the seed region, which is usually situated at nucleotides 2 to 7 of the miRNA's 5′ end. The binding results in an inhibition or abrogation of the translation process. It is estimated that up to 60% of all protein-encoding genes are subject to miRNA-based post-transcriptional regulation [106], making miRNAs central regulators of cellular signaling with a widespread impact on almost every biological process [6]. Besides their effects on the post-transcriptional level, there is recent evidence that miRNAs can translocate to the nucleus to regulate the transcription efficiency of specific genes, further enhancing their impact on cellular signaling networks [107].

How to modify cellular miRNA expression?

The general aim of miRNA therapeutics is to modify and ideally reverse pathological miRNA expression changes. This includes the enhancement or reconstitution of endogenous miRNAs that act as pathological suppressors and the expressional reduction or functional blocking of miRNAs that act as pathological drivers. To modify miRNA levels, nucleic acids are commonly used (Figure 1), including synthetic miRNAs (**miRNA mimics**), recombinant expression vectors carrying miRNA encoding sequences, and oligonucleotide-based miRNA inhibitors (**anti-miRs**) [8].

One of the currently pursued advanced approaches makes use of small cell permeable molecules. These small molecules exert their function by, for example, the interaction with proteins involved in the process of miRNA biogenesis or via binding to miRNA-specific secondary structures [9]. Small molecules are designed with the aid of bioinformatics tools or are identified through experimental screening of pharmacologically active chemical compounds [10,11]. A recent example is the identification of an inhibitor for the oncogenic miR-21. This inhibitor was identified by a target-oriented screening of various low-molecular-weight chemical compounds [11]. Natural compounds are also a rich source for miRNA interfering molecules [12]. Curcumin has, for example, been shown to act on multiple miRNAs to inhibit breast cancer cell growth [13].

A further strategy toward the development of miRNA therapeutics is to combine miRNA-based approaches together with treatments by conventional drugs. The efficiency of drug-based therapies can particularly be improved by miRNA-based interventions that target cellular pathways, which affect therapeutic outcomes [14]. Liver-specific miR-122 is considered as a driver of hepatitis C virus (HCV) infection and maintenance in hepatocytes [15]. In a Phase II clinical trial (ClinicalTrials.gov identifiers NCT01200420, NCT01872936), resistance against HCV treatment has been counteracted by combining conventional viral protein inhibitor drugs with the miR-122 inhibitor miravirsen/SPC3649 [16]. Combined application schemas of chemotherapeutics and miRNA manipulators are especially being developed for the improvement of antitumor therapies, including therapies of common cancers such as breast cancer [17,18].

The combined use of miRNAs with **siRNAs** offers another route to improve the efficiency of therapeutic miRNAs. siRNAs constitute a group of small RNAs conceived for the specific regulation of a single or few target genes [19]. The establishment of siRNA drugs is in general more advanced than miRNA-based drugs [20]. A combination of miRNAs and siRNAs can be achieved by coexpression in a recombinant plasmid, as recently shown for human lung cancer cells [21].

Glossary

amiRNAs: artificially designed RNA constructs that combine the target specificity of siRNA sequences with the processing capacity of primary miRNA scaffolds. amiRNAs are used as recombinant expression constructs in therapeutic approaches.
Anti-miRs: a subgroup of ASOs that target endogenous miRNAs to mediate their degradation or functional blocking. They are used in therapeutic approaches to inhibit miRNAs, which act as pathological drivers.
Antisense oligonucleotides (ASOs): single-stranded oligonucleotides that hybridize to complementary sequences of endogenous RNA species to induce an inhibitory effect.
Aptamer: a single-stranded oligonucleotide that is artificially designed to act as a high-affinity ligand of cellular surface receptors. Aptamers can be conjugated to miRNA-based therapeutics to mediate directed uptake by specific tissues or cell types.
Locked nucleic acid (LNA): methylene bridge modification that locks the ring flexibility of nucleotide bases. In therapeutic approaches, LNA-modified bases are used to promote the stabilization and the cellular uptake of oligonucleotides. They can also enhance the capacity of anti-miRs to associate with their targeted miRNAs.
miRNAs/miRs: a class of small noncoding RNAs that regulate protein-encoding genes at the post-transcriptional level. Deregulated miRNA expression causes changes in cellular signaling networks in various diseases.
miRBase: a database providing information on experimentally verified and annotated miRNA sequences.
miRNA mimics: double-stranded RNA oligonucleotides that mimic endogenous miRNA duplexes. They are used in therapeutic approaches to enhance or reconstitute endogenous miRNAs that act as suppressors of, for example, tumors.
miRNA sponges: (circular) RNAs with multiple binding sites deflecting miRNAs from their endogenous mRNA targets. miRNA sponges are used as artificial expression constructs in therapeutic approaches to functionally inhibit miRNAs.
N-acetylgalactosamine (GalNAc): biomolecule that induces endocytosis through asialoglycoprotein receptors.

Artificially designed miRNA constructs, referred to as '**amiRNAs**,' promise further advancement toward therapeutic miRNAs. amiRNAs are combinations of siRNA sequences and scaffolds of primary miRNA transcripts. While amiRNAs show high target specificity, because of their siRNA-based design, their cellular processing is ensured by their endogenous miRNA-based structure [22]. An amiRNA-based drug (AMT-130) that includes a siRNA sequence against the *Huntingtin* gene together with a pri-miR-451 scaffold is currently being employed in a clinical trial on Huntington's disease (ClinicalTrials.gov identifier NCT04120493) [23–25].

miRNA sponges offer an option to manipulate cellular levels of miRNAs. These are RNA constructs harboring multiple miRNA binding sites. miRNA sponges exert their function through sequestration of endogenous miRNAs. The effectiveness of miRNA sponges, including circular RNAs, has been analyzed in several studies. The expression of an artificially designed circular RNA sponge, including six alternating binding sites for the inhibition of miR-132 and miR-212, has been tested, for example, on mouse models for the treatment of cardiovascular diseases [26]. Additional studies highlight the potential of naturally occurring transcripts as a source for therapeutically usable miRNA sponges. Recently, the circular RNA hsa_circ_0120472, which includes two predicted miRNA binding sites, has been shown to act as an efficient sponge to inhibit miR-550a in human breast cancer cells [27]. The yet increasing number of new strategies that are currently being pursued to modify and reverse pathological miRNA expression changes will certainly promote the development of therapeutic approaches.

What are severe side effects of miRNA therapeutics?

Depending on the chosen route of administration and the way to warrant an intracellular delivery, the effects of miRNA therapeutics are not necessarily restricted to the intended tissue or cells but can also cause systemic side effects. A prominent example of the occurrence of disastrous side effects is MRX34, a synthetic miR-34a mimic. A clinical study with MRX34 for tumor treatment (ClinicalTrials.gov identifier NCT01829971), including various solid tumors and hematologic malignancies, had to be terminated prematurely because of severe immune-related side effects causing the death of four patients [28,29]. The trial was designed to make use of the properties of miR-34a as a powerful tumor suppressor [30]. The miR-34a mimic was systemically administered by a liposomal amphoteric (i.e., pH-dependent) delivery strategy, which was supposed to take effect specifically in the low-pH environment of tumorous tissues [31]. Animal models, however, showed an miR-34a mimic uptake not only in tumorous tissues but also in bone marrow and spleen [32,33], both of which are known to be involved in the generation and preservation of immune cells. Accordingly, in context with the clinical testing, a dose-dependent modulation of several target genes was observed in white blood cells [28]. It is now evident that miR-34a not only functions as a tumor suppressor but also impacts the signaling of immune cells, for example, by regulating calcium or chemokine signaling [34,35]. Although the causative link between the fatalities and the miR-34a function in immune cells awaits confirmation, the adverse effects of MRX34 nevertheless highlight the need for *a priori* risk assessment with a special focus on the *in vivo* targeting of miRNA therapeutics. It has to be acknowledged that later studies did not report comparable severe side effects, as addressed below. In the following sections, we describe emerging concepts currently examined for administration and cellular delivery of potential miRNA therapeutics.

How to administer miRNA therapeutics?

Up to now, clinically tested miRNA drug candidates are mostly administered either via skin or intravenous injection (compare Table 1). For respiratory diseases, miRNA therapeutics can also be administered by inhalation, which has already been tested in clinical trials for other RNA-based therapeutics, particularly siRNA- and mRNA-based therapeutics [36].

GalNAc can be conjugated to miRNA-based therapeutics to mediate uptake by liver cells.

RNA induced silencing complex (RISC): RNA-incorporating protein complex that functions in reducing protein expression. The RNA component that can be an miRNA that binds to a complementary mRNA, which is subsequently either degraded or the translation of which is inhibited.

siRNAs: a class of small non-coding RNAs conceived for the regulation of a single or few target genes. SiRNAs and their combination with miRNA-therapeutics are central to therapeutic applications.

3′ Untranslated region: region at the 3′-hydroxyl directional end of mRNA molecules that is downstream of the stop codon. miRNA targeted sequences are usually located within 3′ untranslated regions.

Table 1. Clinical trials with miRNA therapeutics[a]

miRNA drug name	Targeted miRNA	Mode of action	Background disease	Body application/permission of cellular uptake	Clinical trial number(s)	Refs
AMT-130[b]	Artificial miRNA	amiRNA expression	Huntington disease	Stereotaxic infusion/viral transfer (adeno-associated vector)	NCT04120493	[23–25]
RG-012/lademirsen/SAR339375[c]	miR-21	Anti-miR	Alport syndrome	Subcutaneous injection/chemical modification (phosphorothioate)	NCT03373786, NCT02855268	[70,76,122,123]
RG-125/AZD4076[d]	miR-103/107	Anti-miR	Nonalcoholic steatohepatitis (NASH) in patients with type 2 diabetes/prediabetes	Subcutaneous injection/biomolecule conjugation (GalNAc)	NCT02612662, NCT02826525	[76–78]
MRG-110[d]	miR-92a	Anti-miR	Wounds	Skin injection/chemical modification (LNA)	NCT03603431	[124,125]
MesomiR 1[d]	miR-16	miRNA mimic	Malignant pleural mesothelioma, non–small cell lung cancer	Intravenously/vehicle transfer (nonliving minicells)	NCT02369198	[56,57,126]
CDR132L[d]	miR-132	Anti-miR	Heart failure	Intravenously/chemical modification (LNA)	NCT04045405	[127,128]
Remlarsen/MRG-201[e]	miR-29	miRNA mimic	Keloid disorder	Skin injection/biomolecule conjugation (cholesterol)	NCT02603224, NCT03601052	[73,103,104]
Miravirsen/SPC3649[e,f]	miR-122	Anti-miR	Chronic hepatitis C virus	Subcutaneous injection/chemical modification (LNA)	NCT02508090, NCT02452814, NCT01200420, NCT01872936, NCT01727934, NCT01646489	[16,129–132]
MRX34[g]	miR-34a	miRNA mimic	Solid tumors (e.g., hepatocellular carcinoma), melanoma	Intravenously/vehicle transfer (liposomal)	NCT01829971, NCT02862145	[29,32,133]
RG-101[g]	miR-122	Anti-miR	Chronic hepatitis C virus	Subcutaneous injection/biomolecule conjugation (GalNAc)	EudraCT numbers 2015-001535-21, 2015-004702-42, 2016-002069-77	[76,79,80]
Cobomarsen/MRG-106[g]	miR-155	Anti-miR	Mycosis fungoides	Intravenously/chemical modification (LNA)	NCT02580552, NCT03713320, NCT03837457	[134–136]

[a]NCT numbered trials are registered at ClinicalTrials.gov; EudraCT numbered trials are registered at EU Clinical Trials Register (clinicaltrialsregister.eu).
[b]Phase I ongoing.
[c]Phase II ongoing.
[d]Phase I completed.
[e]Phase II completed.
[f]Unknown status.
[g]Stopped/terminated.

Besides these straightforward protocols, more elaborate procedures are currently being explored for the administration of miRNA-based drugs (Figure 2A, Key figure). A promising approach is the embodiment of miRNA therapeutics into a biodegradable 3D matrix, which can be implanted, for example, into an affected tissue as part of a surgical intervention. Current experimental

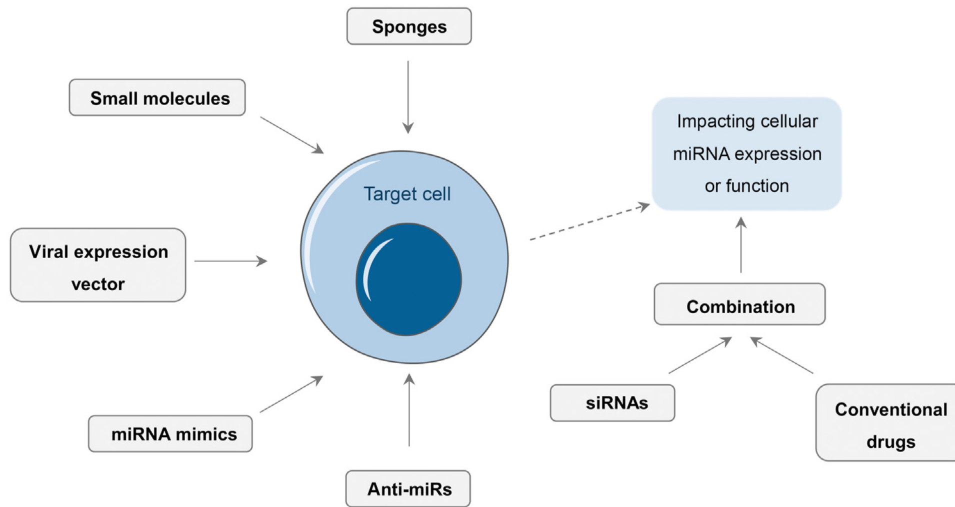

Figure 1. Schematic overview on routes for miRNA targeting in the cell. Therapeutic miRNA manipulations can target the expression or function of pathologically relevant miRNAs via anti-miRs mediating degradation or functional blocking of endogenous miRNAs, synthetic miRNA mimics imitating endogenous miRNA double-strands, viral transduction of miRNA expression constructs, small molecules interfering with miRNA biogenesis, or miRNA sponges causing functional inhibition by deflecting endogenous miRNAs from their mRNA targets. In addition, a combined use with either siRNAs or conventional drugs is employed in current clinical and preclinical approaches.

approaches in mice show that implanted 3D matrices can be used to induce a continuous and tissue-related release of miRNA-based curatives [37,38]. Rapid progress is to be expected because 3D matrices are also optimized for the delivery of other nucleic acid–based therapeutics and conventional drugs with wide-ranging design features, including different application routes, such as edible or injectable carriers (reviewed elsewhere [39–41]).

A very recent concept envisages the oral administration of miRNA-based therapeutics that stem from plants. This dietary application is based on the idea of a therapeutic value of miRNAs that originate from medically relevant plant sources [42]. However, there are contrary findings regarding the bioavailability and the in-human functionality of miRNAs contained in plant food. Among others, the target gene regulation in mammalian cells by plant miRNAs, such as the cross-kingdom regulation of LDLRAP1 by the plant MIR168a, remains a matter of discussion [43,44]. Another open question concerns the origin of plant miRNAs that are found in mammalian tissues or body fluids and that may result from contamination [45,46]. In light of the controversies in this field, further investigations are needed before any therapeutic application can be implemented [47,48].

To ensure the intended effect in the final target cell, various challenges need to be overcome, including, among others, a protected transport in the blood, a specific organ targeting, and a fine-tuned dosing. To bypass these problems in part, miRNA-based therapeutics could be applied *ex vivo* to specific cell types such as blood cells. miRNA therapeutics offer themselves, for example, to promote the effectiveness of adoptive immune cell transfer approaches by impacting genes involved in T cell activation, fitness, or effector function (e.g., as reviewed by [49]). miRNA manipulations (i.e., miR-155 enhancement and miR-146b inhibition) have recently been shown to potentiate adoptive T cell treatments [50,51]. In general, the application of miRNA-based drugs, as part of an *ex vivo* manipulation, bears the promise of most immediate clinical effect.

Key figure

Schematic overview of administration routes and features of miRNA therapeutics

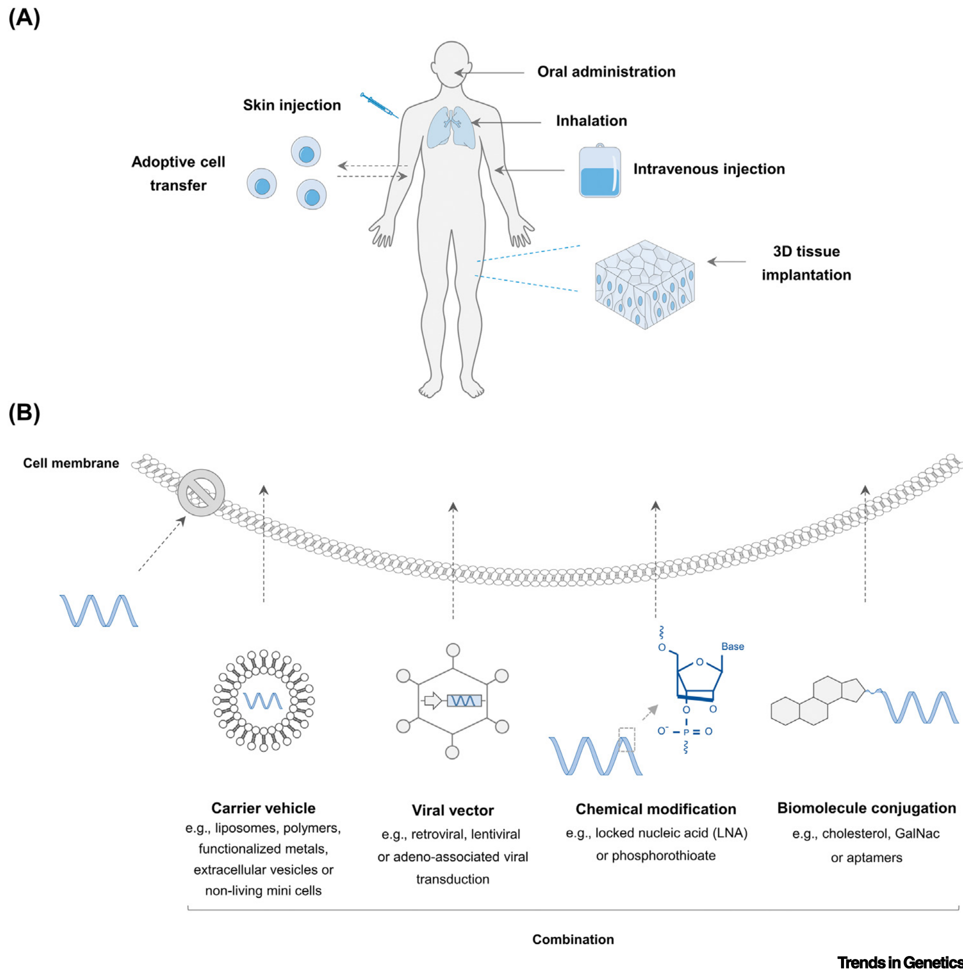

Figure 2. (A) miRNA therapeutics can be applied by systemic administration (e.g., by venous injection) or by cell-/tissue-directed administration, extracorporeal miRNA approaches, adoptive cell transfer, or implantation of 3D matrices releasing miRNA therapeutics. (B) Nucleic acid–based miRNA therapeutics show a low cell membrane permeability. Efficient intracellular delivery can be achieved by nano-sized carrier vehicles, viral transduction, introduction of chemical modifications, or conjugation to biomolecules that further a receptor-mediated uptake. Abbreviations: GalNAc, N-acetylgalactosamine; miRNA, microRNA.

How to achieve *in vivo* cellular targeting and efficient uptake of miRNA therapeutics?

Cellular membranes show a low permeability for nucleic acids, including miRNAs, because of their hydrophobic and anionic character. Various strategies have been developed to improve the cellular uptake of RNA-based therapeutics (Figure 2B). This can be achieved by the use of nano-sized carriers, virally transduced artificial expression, or tailoring therapeutic oligonucleotides via the introduction of chemical modifications or the addition of biomolecule conjugates [52,53].

Therapeutic intracellular miRNA delivery can be accomplished by carriers, including liposomes, polymers, nanocomplex-forming functionalized metals, extracellular vesicles (EVs), or nonliving minicells [52,54]. Common to these approaches is a carrier binding or a packaging of the nucleic acid and, as a consequence, the masking of their negative charge and the protection against RNA degradation [53,55]. The strategy of a liposome packaged miRNA has already been applied in clinical studies, as is the case for the delivery of MRX34 (ClinicalTrials.gov identifiers NCT01829971, NCT02862145) [28,29]. Likewise, bacterial minicells have found their way into a clinical setting, specifically in a Phase I study on miR-16 mimics (MesomiR 1; ClinicalTrials.gov identifier NCT02369198) [56,57]. Although the therapeutic use of miRNA-loaded bacterial minicells has generally been considered safe, studies have also described side effects such as dose-limiting toxicities, decreased lymphocyte counts, or cardiac events [57]. EVs are increasingly used as potent and well-tolerated drug transfer vehicles [58]. They can be derived from an endogenous cell source and can be loaded with a desired cargo to permit in-body cellular transfer [58]. A recent study showed, for example, that EVs derived from mesenchymal stromal cells which originate from human adipose tissue can be genetically engineered and used to package miR-125b. Corresponding constructs were capable of inhibiting the proliferation of human hepatocarcinoma cells [59].

Nonpathogenic viral vectors offer a further option for intracellular delivery of miRNA-based therapeutics. Not least because of their use for coronavirus disease vaccines, viral vectors have become a major focus of attention [60]. Recombinant viral vectors are genetically modified to enter the cell and to subsequently induce the expression of cloned sequences encoding the intended RNA. While a retroviral delivery brings about a risk for a genomic integration, adeno-associated viral delivery systems largely ensure that the transduced recombinant remains transiently stable in a transcriptionally active episomal form in the host cell's nucleus [61,62]. An adeno-associated viral vector system is currently employed in a Phase II study on the treatment of Huntington's disease to permit the cellular delivery of the amiRNA drug AMT-130 (ClinicalTrials.gov identifier NCT04120493) [23–25]. Although viral delivery systems are unquestionably powerful tools to further cellular uptake and expression of miRNAs, various complications such as immunogenicity have been reported [63]. The outcome of clinical trials such as the one for amiRNA drug AMT-130 must be awaited before a further judgment can be drawn.

Chemical modifications offer a possibility to enhance the cellular uptake of oligonucleotides [53,64]. Modifications of the phosphate backbone, the nucleobases, or the ribose sugar can mask the charge of the miRNAs and further their adhesion to the cell surface, thereby facilitating the cellular uptake [53]. Common modifications are **locked nucleic acid (LNA)** bases [64] that are characterized by the introduction of methylene bridges to reduce the ribose ring's flexibility for a locked conformation of the modified nucleotides [65,66]. LNA-modified RNAs are stabilized against ribonucleases and more easily taken up by the cell through an as yet poorly understood endocytosis mechanism [67]. LNA modifications are often applied to single-stranded anti-miRs [e.g., **antisense oligonucleotides (ASOs)**]. The locked conformation enhances the capacity of ASOs to form stable duplexes by binding to and blocking the targeted miRNA [64,67]. LNA-modified oligonucleotides have become central to strategies for miRNA inhibitory therapeutics (compare Table 1). In addition, phosphorothioate modifications that insert a sulfur atom into the phosphodiester backbone of the oligonucleotide have emerged as a promising approach to enhance oligonucleotide stability and facilitate endosomal uptake by stabilin receptors expressed on cell surfaces (e.g., of kidney cells) [68,69]. The latter strategy has been applied for the renal delivery of synthetic miR-21–anti-miR (RG-012/lademirsen/SAR339375) in a clinical study on Alport syndrome (ClinicalTrials.gov identifiers NCT03373786, NCT02855268) [70]. However, some

sequence-independent effects have been reported in context with phosphorothioate-modified oligonucleotides [71].

Perhaps the most promising option to efficiently direct the cellular uptake of therapeutic oligonucleotides is offered by the covalent conjugation of biomolecules such as lipids, peptides, or sugars that work via receptor-mediated endocytosis mechanisms [53]. An example of a biomolecule conjugate for a cell type–independent delivery is lipophilic cholesterol [72] that has been used to facilitate the uptake of a skin-injected miR-29–based mimic (remlarsen/MRG-201) by human skin fibroblasts in a clinical Phase II study on keloid disorder (ClinicalTrials.gov identifiers NCT02603224, NCT03601052) [73]. However, biomolecule conjugates also allow directed targeting if their uptake is mediated through receptor interactions associated with specific cell types. **N-acetylgalactosamine (GalNAc)** is a prominent example of a biomolecule conjugate that promotes a targeted uptake of miRNA-based therapeutics via endocytosis by the stimulation of the liver cell–specific asialoglycoprotein receptors [74,75]. GalNAc was used, for example, as a conjugate to an miR-122 inhibitor (RG-101) and to an miR-103/107 inhibitor (RG-125/AZD4076) in clinical trials on chronic HCV [EU Clinical Trials Register (clinicaltrialsregister.eu) EudraCT numbers 2015-001535-21, 2015-004702-42, 2016-002069-77] and steatohepatitis (ClinicalTrials.gov identifiers NCT02612662, NCT02826525), respectively [76–78]. The clinical trial on RG-101 was interrupted, however, because of reports of side effects (i.e., jaundice cases) the cause of which is still to be elucidated [76,79,80]. Further progress regarding the biomolecule-mediated delivery of miRNAs is likely to be achieved by the development of **aptamer** conjugates. Aptamers are single-stranded nucleic acids developed by rational design approaches as high-affinity ligands of cellular surface receptors, facilitating their intracellular uptake by receptor-mediated transport [53,81]. Although the design phase of potent aptamers can be demanding, the aptamers have the advantage of being readily produced by standard *in vitro* synthesis techniques and able to be coupled to corresponding miRNA therapeutics by simple sticky-end annealing [81]. Aptamer-conjugated miRNA therapeutics, such as the GL21.T-miR-34c conjugate, are currently being tested in preclinical studies to achieve a selective targeting of tumor cells, including human lung cancer cells [82].

In the rapidly evolving field of miRNA delivery techniques, combinations of different strategies have increasingly been tested to further enhance the therapeutic effectiveness and specificity of the cellular targeting. Promising results were obtained, for example, in preclinical studies for LNA modifications or nano-carrier vehicles in combination with cell type-specific biomolecule conjugates [83,84].

How to ensure functional impact in the cell?

Beyond the need for finding the best suited administration routes and an improved cellular targeting, the establishment of successful miRNA-based therapeutics requires a comprehensive understanding of their molecular effects. The decisive question for any miRNA-mediated therapy is, how does an exogenous miRNA therapeutic ultimately work in the individual cell? To achieve a desired effect, one has to acknowledge the rather complicated mechanisms of action of miRNAs. Single miRNAs not only can regulate extended subsets of mRNA targets but also are vice versa frequently targeted by multiple miRNAs [6]. The pivotal role of miRNAs with their pleiotropic effects in regulatory networks are considered as major challenges for miRNA-based therapeutic approaches [85]. A detailed and comprehensive functional characterization of single candidate miRNAs is a necessary prerequisite for their therapeutic use. Toward this characterization, many hurdles need to be overcome, ranging from the confirmation of the authenticity of an miRNA as a true miRNA to improved miRNA target prediction algorithms and the implementation of experimental strategies that allow an efficient validation of a larger number of targets (Box 2).

Box 2. Computational tools for predicting downstream effects of miRNAs

For predicting miRNA gene interactions, several computational tools exist, spanning several scales [88]. First, target predictors for single miRNAs were developed, including TargetScan [108] or miRanda [109]. Respective tools suggest target genes that are the basis for modeling effects of miRNAs on genes and help to select the best candidate targets for validation but can partially lack specificity or sensitivity in predicting the actual targets. From the predicted and validated targets, using expression data and/or sequencing information, more systemic analysis tools were developed to model the mutual influence of miRNAs on genes and pathways [110]. For example, the program miRTarVis [111] displays coexpression networks of paired miRNA and mRNA data, MIENTURNET [112] generates interaction networks of miRNA and mRNA with enrichment analysis, miRViz [113] visualizes networks for multiple species, miRNet [114] supports statistical analysis and facilitates exploration of miRNA–target interaction networks, miTALOS [115] analyzes miRNA function in a tissue-specific manner, and miRTrail [116] analyzes miRNA and gene expression data in an integrated manner. To increase the specificity of target predictions *a priori*, available information from pathway databases can be used. It is known that target genes of miRNAs can orchestrate entire pathways. By adding the information which putative target genes are enriched on functional biochemical networks, the validation rate of target predictors increases significantly [90]. Finally, to generate a systematic analysis of miRNAs in the context of target genes or vice versa, tools that incorporate validated and predicted targets, target pathways, and other information are developed. One example is miRTargetLink2 [117] using miRNA gene associations from published repositories such as miRPathDB2 [118] or mirDIP [119]. To improve the prediction of functional effects, respective tools frequently use existing application programming interfaces to web services and online tools for *in silico* pathway analysis of miRNAs and genes contained in the interaction graph [120,121]. An overview of miRNA target analysis tools has been published recently [88].

Expression analyses by high-throughput approaches such as next-generation sequencing can provide the first evidence for therapeutically promising miRNA candidates [86]. Surprisingly, only a relatively small number of miRNAs have been characterized comprehensively and validated for their nature as true miRNAs [1]. The problem of falsely annotated miRNAs and those identified only by low-quality sequence data has been acknowledged by **miRBase**, which separately lists high-confidence entries in its recent releases. Out of a total of 2349 entries in the latest version of miRBase (version 22.1), 897 sequences are considered as high confidence; that is, they are considered as bona fide human miRNAs. There are different criteria that contribute to this assessment, including, for example, the presence of common miRNA structural elements [2]. Although it is evident that an erroneously annotated miRNA should not be employed in a therapeutic assay, the still large number of low-confidence miRNA entries in databases should prompt the greatest caution when designing therapeutic approaches with recently identified miRNAs. A therapeutically envisaged application of miRNAs should be designed solely with those that have been experimentally validated by independent methods, such as PCR or preferentially northern blotting, ideally using endogenous miRNAs or exogenously applied and overexpressed miRNA precursors [1,87].

Besides the identification and confirmation of miRNA candidates for a therapeutic modification, the challenge of identifying their targets remains an ever greater obstacle [88]. The *in silico* prediction based on properties and characteristics of the miRNA seed binding, and the analysis of putative target genes and pathways, can be achieved by using various bioinformatics tools such as Tools4mirs, a metarepository that lists target prediction tools and toolboxes for the functional analysis of targets [89]. Experimental evidence on the predicted miRNA target interactions frequently stems from reporter assays. The **3′ untranslated region** of the gene of interest is cloned downstream of the luciferase gene, and the resulting recombinant plasmid is cotransfected with miRNA mimics or miRNA inhibitors. Functional binding of the miRNA or an inhibitor can be measured by reduced or increased luciferase activity [86]. Major progress has recently been made by the establishment of an automated and standardized high-throughput miRNA interaction reporter assay, which allows target testing in context with cellular pathways [90]. There are, however, several caveats of these reporter assays, including nonphysiological amounts of the exogenous miRNA or the inhibitor and a cellular background (e.g., model cells) that is different from that cell, where the interaction between the miRNA and its mRNA target takes place. Additional

evidence for miRNA targets can be provided by an inverse relationship between the levels of miRNA expression and those of the target mRNA and/or its encoded target protein in the cells of interest [91,92]. Further confirmation of true miRNA targets can be generated by transfecting miRNAs or inhibitors in cells of interest and measuring the effects both on mRNA targets and on proteins encoded by these mRNAs [86]. Admittedly, an efficient transfection may not be achieved for many cell types.

In addition to the reporter assay-based approaches, mRNA targets can be identified by immunoprecipitation of the Ago complex and subsequent sequencing of bound miRNAs and mRNAs. Various modifications, such as high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation, photoactivatable ribonucleoside-enhanced crosslinking immunoprecipitation, or crosslinking, ligation, and sequencing of hybrids, increased the efficiency of this approach [86]. Major limitations of immunoprecipitation-based approaches are the requirement of cell culture systems and the need for a large number of cells. Functional assays depending on the targeted pathway can finalize the chain of evidence for true miRNA–mRNA target relationships [86]. The *in vitro* manipulation of miRNA expression by recently developed CRISPR-Cas9-based genome editing techniques (i.e., the generation of cellular miRNA-knockout models) can help to confirm causative links between an miRNA, its target interactions, and the resulting phenotypes [93,94].

This above analysis scheme not only is required to confirm the authenticity of miRNA–mRNA target relationships but also helps to identify indirect miRNA effects. Each step of the scheme, including the functional approaches, the immunoprecipitation, next-generation sequencing, and high-throughput proteomics, can identify off-target effects, which are to be expected due to the pleiotropic effects of miRNAs. Toward the development of miRNA therapeutics, further attention has to be given to the cellular background, the regulatory effects of nucleus-resident miRNAs the functionality of which is as yet poorly understood [6], and the dose dependency of miRNA–target interactions [95,96].

How to deal with dosing?

As for any exogenous RNA, the dosing of miRNA therapeutics is usually beyond the physiological range of endogenous miRNA expression and can consequently cause unpredictable off-target effects [97,98]. Because there is a dose dependency of miRNA–target interactions, the dosage of miRNA therapeutics directly affects the genes that are targeted by the manipulated miRNA. Animal models show that, depending on the dosing, manipulated miRNA expression levels can cause outcomes that are contrary to the therapeutic objectives [95,96]. The dosage of miRNA therapeutics can also affect genes that are not directly targeted by the manipulated miRNA. Because miRNAs compete for cellular resources such as free **RNA-induced silencing complexes (RISCs)**, therapeutic overexpression of a single miRNA, for example, can oust other endogenous miRNAs from the RISC. This in turn can trigger potentially toxic off-target effects on multiple signaling pathways [93,99,100].

Hence, the dosing of miRNA therapeutics has to be kept within a given range to induce a therapeutic effect [101]. There is, however, a lack of data describing the dose-dependent target gene regulation in a quantified manner. First evidence shows that the overall quantitative range of cellular miRNA expression changes range in a magnitude of approximately 10^3 copies per cell. Additional quantifying studies for both physiological and pathological conditions are needed to further the pharmacokinetic characterization of potential miRNA therapeutics [76,91].

There are different strategies to achieve an appropriate dosing in therapeutic approaches. A combined use of cooperating miRNAs offers a potential strategy to keep the therapeutic doses of

individual miRNAs as low as possible [98]. Another way forward is the adoption of vector-based expression systems, designed to warrant physiologically adapted and tissue-specific expression levels using endogenous promoter sequences [22,102].

The problem of dosing also includes the question of how much miRNA has to be applied (i.e., by injection, inhalation, or other topical means) to finally ensure a desired cellular effect. A comprehensive understanding of the quantitative aspects is required for the transport routes from the site of application to the receipt cell. Although less demanding, the extracorporeal use of miRNA therapeutics also requires a detailed quantitative understanding of the miRNA–mRNA interaction. The extracorporeal application of miRNAs requires new methods that are well adapted to a given cell type to ensure the intended miRNA uptake while minimizing side effects due to the employed methods.

Concluding remarks

Although several obstacles need to be overcome in the push toward a clinical routine application of therapeutic miRNAs, these hindrances can be clearly defined and specifically addressed. Important issues to be resolved include the question of how correct targeting can be ensured, how immunogenic reactions can be reduced, and what dosing is required to achieve the desired effect while minimizing side effects (see Outstanding questions). There are already numerous studies on new or optimized methods for the administration of therapeutic miRNAs to the body, the protected delivery in the blood, the directing of miRNAs to target cells, the efficient uptake by target cells, and an optimized gene targeting in the cell. Nevertheless, one must acknowledge that, despite these advances, we are only at the beginning of a process that will finally allow the full benefit of the undoubtedly very high therapeutic effectiveness of miRNAs. Outstanding issues include the dosing required for specific application methods.

Declaration of interests

The authors have no interests to declare.

Outstanding questions

How can therapeutic miRNAs be efficiently directed *in vivo* to their target cell? How can oligonucleotides and transfer vehicles be designed to prevent or at least minimize effects on nontargeted cells? How can a correct targeting be ensured for RNA interfering molecules such as small cell permeable molecules?

Does the use of different carrier materials for an advanced miRNA delivery increase the risk of incompatibilities (i.e., unwanted interactions between the materials and miRNA therapeutics)? Does the combined use of miRNAs and conventional drugs bear the risk of incompatibilities?

To what extent do immunogenic reactions occur due to viral transfer systems? Can severe immunogenic reactions also be caused by miRNAs modifications such as LNA miRNAs and amiRNAs, miRNA interfering molecules such as small cell permeable molecules, application systems such as biodegradable 3D matrices, carriers such as functionalized metals, viral transfer systems, or biomolecule conjugates such as aptamers. Can immunogenic reactions be reduced by masking reactive components?

Do miRNA modifications such as LNA miRNAs impact the mRNA targeting? To what extent can nonhuman (e.g., plant-derived miRNAs) be used as therapeutic miRNAs in humans?

In case of future frequent use of miRNA therapeutics, how high is the risk for genomic integrations of viral transduced expression constructs carrying miRNA or an miRNA?

How does the expression pattern of endogenous miRNAs and mRNAs influence the effect of exogenous miRNAs, depending on variables such as the cell type, the cell cycle, and the cell environment?

What dosing is required for specific application methods (i.e., for skin injection, infusion, inhalation) and carrier-based methods such as biodegradable 3D matrices? How can dosing be controlled throughout

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complex in-body delivery routes? Can predictable and reproducible rates of cellular uptake be achieved under variable environmental *in vivo* conditions? How can dosing contribute to an intended gene targeting?

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