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microRNAs as Therapeutic Targets to Combat Diverse Human Diseases

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1. Introduction

For decades, control of cellular behavior was thought to be the exclusive purview of protein-based regulators. However, the recent discovery of small RNAs (sRNAs) as a universal class of powerful RNA-based regulatory biomolecules has the potential to revolutionize our understanding of gene regulation in practically all biological functions, as sRNAs have been found in diverse organisms from bacteria to plants to man. A class of sRNAs in eukaryotes, termed microRNAs (miRNAs), has been found to modulate a wide variety of cellular functions, including cell growth, cell differentiation, and apoptosis. miRNAs function as regulators by base-pairing with trans-encoded mRNAs to prevent translation of mRNA into protein at the post-transcriptional level. By modulating the expression levels of target genes, miRNAs enable rapid adaptation of cellular physiology in response to specific environmental changes. It is estimated that at least ~30% of the human genome is regulated by miRNAs. (Lewis, et al., 2005) In this review, we will discuss recent discoveries that implicate miRNA function in host immunity, including specific miRNA expression in immune cells and their regulation of immune cell development, miRNA regulation of innate and acquired immune response, and viral-encoded miRNAs. These recent advances have strong potential to translate fundamental research in miRNA function into clinical applications. We will also describe the challenges in bringing another RNA interference (RNAi) methodology, small interfering RNA (siRNA), to clinical trials. This analysis will serve as a practical roadmap for development of novel miRNA-based therapies to combat infectious disease by reducing the host inflammatory response and the downstream effects of pathogen infection.

2. miRNA biogenesis and mechanism of action

Most miRNAs are transcribed by RNA polymerase II as either polycistronic or monocistronic transcription units called primary miRNAs (pri-miRNAs), in which one or more hairpin structures with ~33bp stem regions and terminal loops are embedded. (Fig. 1) (Lee, et al., 2004) These pri-miRNAs are capped and polyadenylated and can be as long as several kilobases. (Cai, et al., 2004) Hairpin structures embedded within pri-miRNA transcripts are recognized and excised by the microprocessor complex in the nucleus, which consists of the RNase III-like enzyme Drosha and double-stranded RNA (dsRNA) binding protein DGCR8 (DiGeorge syndrome critical region gene 8). (Landthaler, et al., 2004, Lee, et

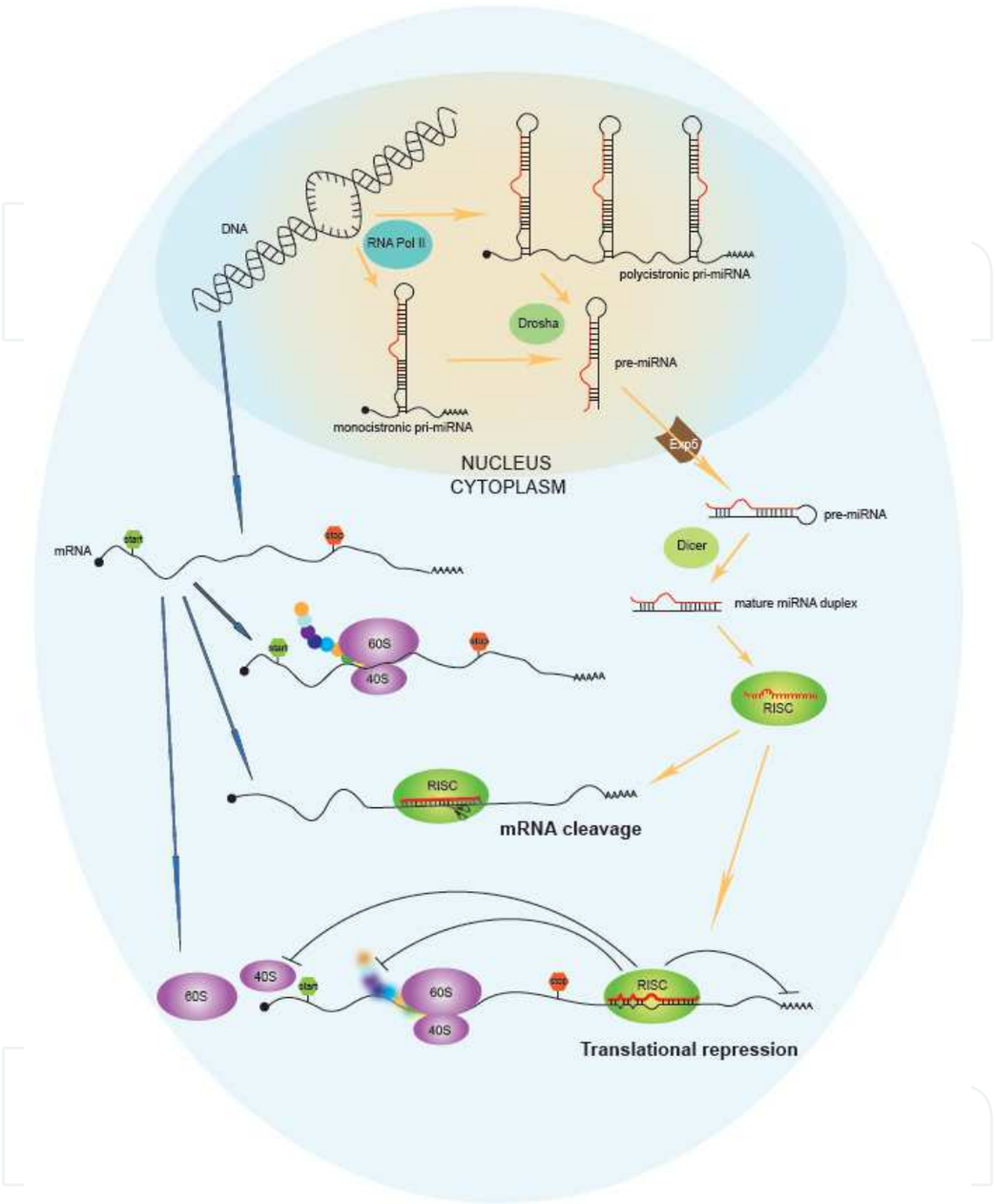


Fig. 1. miRNA biogenesis and mechanism of action. miRNAs are initially transcribed as either polycistronic or monocistronic long primary miRNA transcripts (pri-miRNAs), and then undergo a series of maturation steps including: (1) cleavage by the enzyme Drosha into the intermediate precursor miRNAs (pre-miRNAs), (2) transport from the nucleus to the cytoplasm via Exportin 5, and (3) final processing by the enzyme Dicer into the mature miRNA duplexes for loading into the RNA-induced silencing complex (RISC). Plant miRNAs pair extensively with their target mRNAs, resulting in direct cleavage of the mRNAs. Mammalian miRNAs exhibit partial complementarity with the 3' untranslated region (UTR) of target mRNAs, causing translational repression, which may lead to mRNA degradation.

al., 2003) The released hairpin structure is typically ~65-70 nt and is referred to as a precursor miRNA (pre-miRNA). (Lee, et al., 2002) Pre-miRNAs are then exported into the cytoplasm by Exportin-5 (EXP5) in a Ran-GTP dependent manner. (Yi, et al., 2003) In the cytoplasm, the end opposite to Drosha cleavage in the mature miRNA is cleaved by another RNase III enzyme, Dicer, yielding a 22-25nt duplex. (Hutvagner, et al., 2001, Ketting, et al., 2001) Once the mature miRNA duplex is created, one strand of the duplex is loaded into a multi-protein complex, RNA-induced silencing complex (RISC), to direct subsequent miRNA:mRNA target interaction and gene silencing. (Bartel, 2004, Filipowicz, et al., 2008) This strand is called the guide strand (miRNA), while the other strand is termed the passenger strand (miRNA*), which usually undergoes degradation. There are cases where both strands can mediate subsequent gene silencing. The determination of guide/passenger strand is believed to depend on the thermodynamic stability of the base pairing at the ends of the duplex. The strand whose 5′ end displays less stability will become the guide strand. (Khvorova, et al., 2003) The catalytic component of RISC is the Argonaute (AGO) protein, which mediates binding and silencing of the target mRNAs. (Pillai, et al., 2004)

In general, miRNAs down-regulate translation by binding to the miRNA response elements (MREs) in the 3′UTR (3′ untranslated region) of their mRNA targets to cause inhibition of mRNA translation, and in some cases, mRNA destabilization. (Filipowicz, et al., 2008) Complementarity between miRNAs and MREs have been shown to be near perfect in plants, but only partial in animals. Multiple mechanisms of action have been proposed. AGO proteins in RISC may inhibit translation initiation by competing with eIF4E for mRNA m^7G cap-recognition. (Kiriakidou, et al., 2007, Mathonnet, et al., 2007) Alternatively, RISC may inhibit translation initiation by preventing the assembly of 80S ribosomes via recruitment of eIF6. (Chendrimada, et al., 2007) There is also evidence that RISC can repress translation post-initiation by causing ribosome drop-off or nascent polypeptide degradation during the elongation step. (Petersen, et al., 2006) Finally, miRNAs can accelerate mRNA destabilization. miRNA-associated targets were found to be enriched in P-bodies, compartmentalized cytoplasmic foci where mRNA decay occurs (Sheth and Parker, 2003), and to be associated with deadenylase, decapping enzymes and exonucleases. (Behm-Ansmant, et al., 2006, Liu, et al., 2005)

miRNA target identification in animals is relatively difficult because of imperfect miRNA:MRE complementarity. One important finding is the so-called "seed rule", in which extensive Watson-Crick base paring between the "seed" region (2-7 nt from the 5′ end) of the miRNA and its target, remarkably reduces the number of false positive predictions. (Lewis, et al., 2003, Lim, et al., 2005) The seed rule has been widely applied as the fundamental criteria by most current prediction algorithms to screen for potential miRNA target genes. Nevertheless, considerable evidence exists to argue that the seed pairing is either not required or not sufficient for predicting miRNA:mRNA interactions. (Didiano and Hobert, 2006) Other features within 3′UTRs, in addition to seed pairing, have been demonstrated to be important determinants, including overall thermodynamic stability of miRNA:mRNA duplex, total number of MREs within the 3′UTR, accessibility of the MRE, position of the MRE related to the stop codon, and local AU rich elements. (Hon and Zhang, 2007, Kertesz, et al., 2007, Li, et al., 2008) Thus, current computational target prediction is far from established, and predicted target candidates need to be experimental verified.

Both biogenesis and function of miRNAs are subject to tight regulation. Almost every aspect of miRNA biogenesis, from transcription and processing to subcellular localization and stability, can be regulated in a sequence-specific and cell-specific manner. Such regulation is

believed to be important for many developmental and physiological processes. For example, the transcription factors Myogenin and MyoD1 induce expression of miR-1 and miR-133 specifically during myogenesis. (Rao, et al., 2006) During stem cell differentiation, the levels of pri-let-7 remain constant, while the levels of mature let-7 duplex increase. (Piskounova, et al., 2008) Interestingly, post-transcriptional suppression of let-7 in undifferentiated cells is mediated by its target Lin-28. Lin-28 not only blocks microprocessor cleavage in the nucleus by directly binding to the loop region of pri-let-7, (Piskounova, et al., 2008) but also prevents Dicer cleavage in the cytoplasm by promoting polyuridylation and degradation of pre-let-7. (Heo, et al., 2009) The nuclear export of miRNA is usually the rate-limiting step of miRNA biosynthesis. (Grimm, et al., 2006) Some pre-miRNAs such as human pre-miR-31, pre-miR-128, and pre-miR-105 are retained in the nucleus instead of being processed into mature miRNAs in certain cell types. (Lee, et al., 2008) Finally, although the degradation of miRNAs is not well-understood, the enrichment of guide strands but not passenger strands in the cells clearly indicates the existence of an as-yet unknown mechanism that quickly and selectively turns over these small RNAs. A family of exoribonucleases that degrades miRNAs have been identified in *A. thaliana*. (Ramachandran and Chen, 2008) Furthermore, under certain conditions, miRNA-mediated silencing can be reversed or blocked. (Kedde, et al., 2007, Schratt, et al., 2006) For example, mir-122-mediated repression of CAT1 (cationic amino acid transporter 1) can be alleviated in human cells lines as a response to starvation or other types of cell stress. (Bhattacharyya, et al., 2006)

3. miRNAs and the host immune system

Mammalian systems have developed a complex system of checks and balances to regulate gene expression in order to respond to pathogen infection. In the last several years, miRNAs are increasingly becoming implicated in the regulation of both immune cell development and function. Proper functioning of the immune system requires elaborate control of both innate and adaptive immune response in order to defend against various pathogens while maintaining self tolerance. miRNAs are required for normal immune system function by helping to maintain this balance. miR-146a is upregulated in human monocytes upon exposure to lipopolysaccharide (LPS), a cell wall component of Gram negative bacteria and established activator of innate immunity. Its upregulation is dependent on NF-κB, a key transcription factor that regulates practically all aspects of the innate immune response, such as synthesis of pro-inflammatory cytokines, including TNFα and IL-1β, and regulation of immune cell migration. Interestingly, TRAF6 (TNF receptor-associated factor 6) and IRAK1 (IL-1 receptor-associated kinase 1), two components of the Toll-like receptor 4 (TLR4) signaling pathway that act upstream of NF-κB, were found to be targets of miR-146a. These findings suggest that miRNAs function in the negative feedback regulation of TLR signaling in order to ensure appropriate strength and duration of the innate immune response. (Taganov, et al., 2006) Another inflammatory mediator, miR-155, is induced by LPS (Ceppi, et al., 2009, O'Connell, et al., 2007, Tili, et al., 2007) and nucleic acids, including poly(I:C) (polyriboinosinic:polyribocytidylic acid) and hypomethylated DNA (O'Connell, et al., 2007), implicating function in both bacterial and viral infection. miR-155 is proposed to fine tune inflammatory cytokine production through negative feedback loops by targeting TAB2 (Ceppi, et al., 2009), FADD (fas-associated death domain protein), IKKε (IκB kinase ε), and Ripk1 (receptor-interacting serine-threonine kinase 1). (Tili, et al., 2007)

In addition to innate immunity, the adaptive immune response is also subject to regulation by miRNAs, in particular miR-155. miR-155-deficient dendritic cells exhibited impaired ability in antigen presentation and T cell activation, suggesting its involvement in bridging innate and adaptive immunity. (Rodriguez, et al., 2007) miR-155 restricts Th2 but not Th1 lineage commitment after CD4+ T cell activation (Rodriguez, et al., 2007, Thai, et al., 2007), and is also required for the differentiation and proliferation of regulatory T helper cells, which function to self-limit the immune response. (Kohlhaas, et al., 2009, Lu, et al., 2009) Furthermore, miR-155 was induced in B lymphocytes upon activation and regulates the germinal center response and generation of immunoglobulin class-switched plasma cells. (Teng, et al., 2008, Thai, et al., 2007, Vigorito, et al., 2007) Naïve B lymphocytes express only immunoglobulin M (IgM) isotype antibodies on the cell surface as a result of V(D)J DNA recombination. Upon activation, B lymphocytes undergo somatic hypermutation (SHM), gene conversion (GCV), affinity maturation, and class-switched recombination (CSR) to produce a vast antibody repertoire with increased diversity, higher antigen-binding affinity, and different isotypes. miR-155 has been shown to regulate expression of activation-induced cytidine deaminase (AID), which catalyzes the SHM, GCV and CSR processes by deaminating cytosine to introduce U:G mismatches in Ig genes. (Teng, et al., 2008) Disruption of miR-155-AID interaction in vivo results in quantitative and temporal alteration of AID expression and defective antibody maturation. Another target of miR-155, the transcription factor PU.1, has been reported to be involved in the reduction of IgG1-switched plasma cells in a miR-155 deficient mouse model. (Vigorito, et al., 2007)

Several miRNAs have also been implicated in different immune development processes. miR-223 is activated by the myeloid transcription factors PU.1 and C/EBP (CCAAT/enhancer-binding protein) and has been shown to control granulocyte development. (Fazi, et al., 2005, Johnnidis, et al., 2008) miR-150 is an important regulator of B cell differentiation through targeting of the transcription factor c-Myb. (Xiao, et al., 2007, Zhou, et al., 2007) Finally, miR-181a modulates T cell receptor sensitivity and signaling strength during positive and negative selection, most likely through downregulation of phosphatases. (Li, et al., 2007)

4. miRNAs and viruses

Interestingly, miRNAs have been identified in various members of the herpesvirus family, such as Epstein-Barr virus (EBV), herpes simplex virus 1 (HSV-1), Kaposi's sarcoma-associated herpesvirus (KSHV), and human cytomegalovirus (HCMV), during both the latent and the productive stage of the viral life cycle. These viral miRNAs share the same biogenesis and execution pathways as their cellular counterparts, and downregulate either viral or host mRNAs in order to evade the host immune system or to control the transition from latent to the productive replication stage. (Cullen, 2009) For example, the degradation of EBV DNA polymerase mRNA by miR-BART2 (Barth, et al., 2008), the suppression of HSV-1 immediate early proteins ICP0 and ICP4 by miR-H2-3p and miR-H6 (Umbach, et al., 2008), and the downregulaton of HCMV viral immediate-early protein IE1 by miR-UL112-1 (Murphy, et al., 2008), help to establish and maintain viral latency. KSHV miRNA miR-K12-6-3p contributes to the formation of Kaposi's sarcoma tumors in vivo by downregulation of host gene THBS1 (thrombospondin 1). (Samols, et al., 2007) Downregulation of host MICB mRNA (major histocompatibility complex class I polypeptide-related sequence B), a ligand for natural killer cells, by HCMV miRNA miR-UL112-1 protects infected cells from natural

killer cells. (Stern-Ginossar, et al., 2007) Another host antiviral gene CXCL11 (CXC-chemokine ligand 11), a target of EBV miRNA miR-BHRF1-3, is downregulated to protect infected B cells from being targeted by cytotoxic T cells. (Xia, et al., 2008)

5. Targeting miRNA expression to modulate gene expression

Medical countermeasures that exploit miRNA function have focused on therapies for cancer. Different approaches have been developed to manipulate expression levels of miRNAs and determine downstream effects on disease. (Fig. 2) A number of studies have demonstrated that specific miRNAs exhibit altered expression levels in tumors, and that "normalization" of miRNA expression in cancer cells may have a therapeutic effect. To up-regulate miRNA expression, either miRNA mimics or miRNA expression vectors can be overexpressed in target cells and tissues. Mirna Therapeutics has reported the systemic delivery of mimics for miR-34 and let-7 via a neutral lipid emulsion, as a strategy for miRNA replacement therapy to inhibit tumor growth and metastasis in a mouse model. (Trang, et al., 2011, Wiggins, et al., 2010) Both miR-34 and let-7 are natural tumor suppressors that exhibit reduced expression in different cancers. Introduction of the mimics in mice led to ~60% reduction in tumor area compared to control mice. These results offer a novel therapeutic strategy for cancer by restoring miR-34 and let-7 expression to wild-type levels to reduce tumor growth. Given that miR-34 and let-7 levels are reduced in a number of different cancers, it may be the case that synthetic miRNA mimics can have broad applicability as anti-cancer agents.

Inhibitory agents that down-regulate miRNA expression, termed antagomirs, have been developed to pair with mature miRNAs through sequence complementarity and block miRNA-mediated gene regulation. (Hutvagner, et al., 2004, Krutzfeldt, et al., 2005) Development of antagomirs that contain locked nucleic acids (LNAs), a backbone modification in which the ribose moiety has been locked by an oxymethylene bridge connecting the C(2′) and C(4′) atoms of the ribose, have yielded unusually stable oligonucleotides with high duplex melting temperatures for more robust therapeutic studies. Their relatively small size, high affinity, and potential for systemic delivery without complicated delivery vehicles has established LNA oligos as a favorite platform for design of RNA-based drug candidates. Antagomirs against miR-16, miR-122, miR-192, and miR-194, conjugated to cholesterol, have been intravenously administered in mice, and corresponding miRNA levels exhibited marked reduction in multiple organs and tissues. A systemically administered unconjugated LNA-anti-miR-122 oligonucleotide led to specific, dose-dependent silencing of miR-122 in non-human primates. (Elmen, et al., 2008) This particular anti-miR was composed of 15 nucleotides, which covered the seed sequence in the 3′ UTR of miR-122 and adjacent nucleotides. In a recent study, 7-mer and 8-mer anti-miRs that only targeted the seed sequence exhibited potent inhibition of miR-21, which exhibits elevated levels in a variety of cancers. (Obad, et al., 2011) These short anti-miRs form strong hybrids with their target miRNAs and sterically block miRNA function. Injection of anti-miR-21 into mice yielded sequestration of the hybrid complex in the main organs and resultant upregulation of a miR-21 gene target. Their enhanced stability and relatively small size positions these seed-directed anti-miRs as potentially strong drug candidates to target specific miRNAs or miRNA families that function in disease onset.

Viral miRNAs are potential anti-viral candidates for therapeutic development and can serve as diagnostic markers for viral infection or specific stages of disease. The host miR-122,

which has two recognition sites in the 5′ UTR of the hepatitis C virus (HCV) genome, is required for virus replication. (Jopling, et al., 2005) Use of specific antagonists that target miR-122 resulted in a reduction in HCV in the liver of a primate model, demonstrating the therapeutic utility of this strategy.(Lanford, et al., 2010) The anti-miR-122 treatment provided continued efficacy in the animals up to several months after the treatment period with no adverse effects or evidence of viral rebound. Based on these studies, Santaris Pharma A/S has initiated the first miRNA-targeted Phase 2a clinical trial, based on the miR-122-inhibitory drug, Miravirsen, to assess safety and tolerability in up to 55 treatment-naïve patients infected with HCV. Designed using LNA technology, Miravirsen sequesters miR-122 from HCV, thus inhibiting replication of the virus. Secondary validation studies, including drug pharmacokinetics and effect on viral load, will be assessed. miRNA technology has also been applied to vaccine development for influenza virus. A miRNA-responsive element (MRE) was introduced into the viral nucleoprotein gene to control the level of viral attenuation via miR-124, which targets the MRE sequence. The resulting viruses produced a species-specific vaccine that generated high levels of neutralizing antibodies in the host. (Perez, et al., 2009)

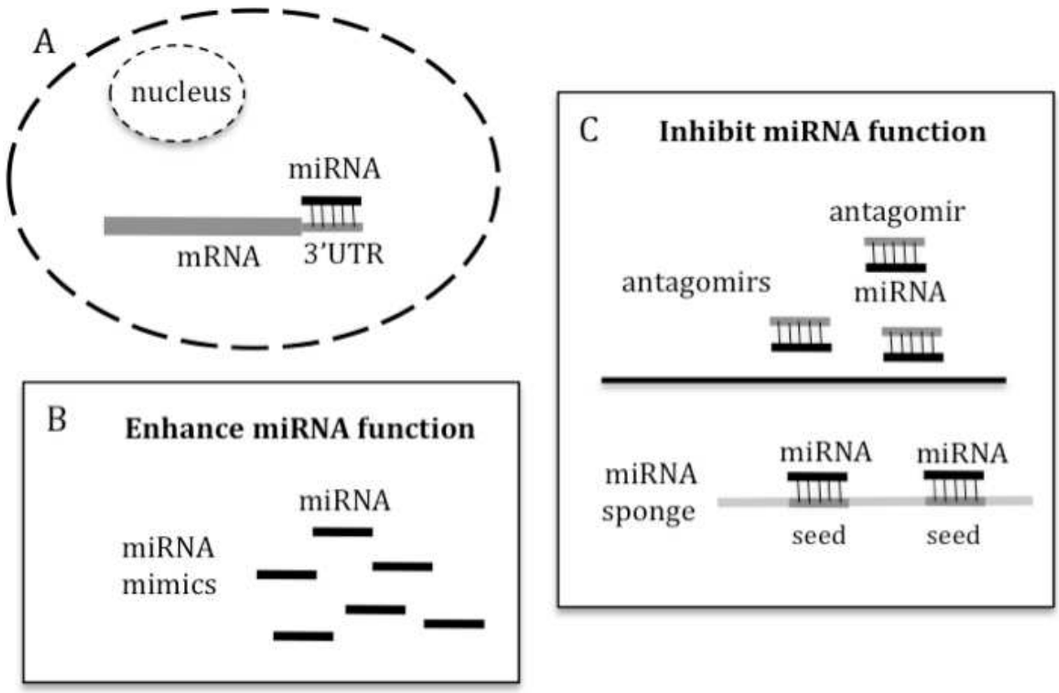


Fig. 2. (A) In general, miRNAs bind to the 3′ UTR of target genes to downregulate gene expression. (B) miRNA mimics provide increased numbers of miRNAs by overexpression or synthetic copies to enhance miRNA function. (C) Inhibition of miRNA function can be achieved by usage of (1) antagomirs, stable complementary oligonucletides, that hybridize to target miRNAs, or with (2) miRNA sponges, which provide alternative binding platforms to levels sequester miRNAs.

Artificial miRNA decoys, termed miRNA “sponges”, provide alternative binding platforms for the miRNAs and inhibit their ability to bind and suppress their endogenous targets. The miRNA sponges often contain a strong promoter to drive expression of binding sites for the

miRNA either in a non-coding transcript or in the 3'UTR of a reporter gene, like GFP. (Brown and Naldini, 2009, Ebert, et al., 2007) Since the interaction between miRNAs and target genes is largely dependent on the seed region of the miRNA, design of the miRNA sponges that incorporate the seed sequence can interact with all members of a miRNA seed family. Both individual miRNAs and large seed families, such as miR-155 and the let-7 family, respectively, have been successfully targeted for continuous loss of miRNA function in multiple mammalian cell lines. (Bolisetty, et al., 2009, Kumar, et al., 2008) Compared to miRNA antisense oligonucleotides, a major advantage of miRNA sponges is the potential for stable integration for continual expression in the genome. Stable transfection of miRNA sponges in cultured cells led to partial derepression of miRNA target gene expression in a variety of cell systems, including mesenchymal stem cells (Huang, et al., 2010) and cancer cell models. (Bonci, et al., 2008, Valastyan, et al., 2009) A high expression level is often required for sufficient inhibition of endogenous miRNAs, given the much lower transgene copy number in stable lines compared to transient plasmid transfection. Forty integrated copies of a miRNA sponge targeting miR-223 driven by a weak promoter were needed to sufficiently suppress miR-223 expression. (Gentner, et al., 2009) Stable expression of sponges also enables miRNA loss-of-function studies that can span days to weeks. After 6 days, neurons that express anti-miR-92 sponges exhibited derepression of a potassium chloride cotransporter and electrophysiological changes in response to GABA treatment. (Barbato, et al., 2010) Both miR-144 and miR-451 were found to be required for erythropoiesis in bone marrow reconstitution experiments 3-4 weeks after transplantation of cells expressing miRNA sponges. (Papapetrou, et al., 2010)

6. RNAi therapeutics: Development of siRNAs for disease therapy

The potential development of new therapies for infectious disease using RNAi-based strategies has attracted the attention of biotechnology entrepreneurs. Multiple small companies have entered the field to transition RNAi from molecular biology tool to next-generation nucleotide-based drugs for treatment of disease. In the last ten years, there have been a growing number of clinical trials based on siRNAs, ~20 bp short interfering RNAs that silence target genes by directing their cognate mRNAs for degradation by the RISC complex. (Davidson and McCray, 2011, Vaishnaw, et al., 2010) Some programs involve local delivery of siRNAs to target tissues, including the eye, kidney, and liver, while others aim to achieve systemic delivery in the body. As with any novel strategy for drug development, there still remain technical challenges that need to be overcome before RNAi-based technology can successfully transition into robust therapies, including minimization of off-target effects (OTE) and systemic delivery of siRNAs in the body. A summary of these challenges and the methods that have been utilized to move the field of RNAi-based therapeutics forward is summarized in Table 1. Given that miRNA-based therapies will most likely face similar challenges that are currently being addressed by the more mature siRNA-anchored drug companies, we provide an overview of the strategies utilized in siRNA-based drug discovery.

6.1 Off-target effects

All RNAi-based therapeutics remain vulnerable to the potential of OTE, in which expression of non-targeted genes is unintentionally modulated. OTE can be categorized into sequence-dependent and sequence-independent effects. In the case of sequence-dependence, the

siRNAs can bind to bystander mRNAs that exhibit partial sequence complementarity but is unrelated to the target gene. This type of OTE can be mitigated through use of bioinformatics screening algorithms to design siRNA sequences that exhibit rare or no direct complementarity to other genome sequences aside from the target gene. In sequence-independent OTE, the siRNAs may have inherent immunostimulatory properties or the endogenous miRNA pathway may be saturated, especially in the case of overexpression of heterologous siRNAs. For example, RNAs have been shown to stimulate the Toll-like receptor (TLR) (Kleinman, et al., 2008) and retinoic acid inducible gene (RIG)-I pathways. (Yoneyama, et al., 2004) Since there does not exist any a priori knowledge about which signaling pathways are activated by specific siRNAs, it may be necessary to evaluate a panel of pro-inflammatory markers to downselect siRNAs with comparatively reduced immunostimulatory properties in preclinical assays. RNAi-based therapies also need to be cautious about the level of exogenous siRNAs expressed in the host. In mouse studies, PolII promoter-driven expression of plasmid-based short hairpin RNA (shRNA) constructs in the liver induced host mortality, which has been attributed to saturation of the transport factor, Exportin 5, that ferries miRNAs from the nucleus to the cytoplasm. (Grimm, et al., 2006) Thus, it is necessary to determine the lowest possible concentration of siRNAs that can still be therapeutically effective for introduction into the host. OTEs and non-specific immunostimulatory responses can also be mitigated using different siRNA backbone modifications, including 2′-O-Me modifications (Jackson, et al., 2006) or DNA substitutions. (Ui-Tei, et al., 2008) Once candidate siRNAs have passed a threshold efficacy level in *in vitro* cell culture studies, leads are then assessed using good laboratory practice (GLP)-compliant preclinical toxicological studies with animal models such as rodents and non-human primates.

6.2 siRNAs enter clinical trials

Alnylam Pharmaceuticals currently has several advanced RNAi-based therapeutic programs that target multiple diseases. Alnylam has completed Phase II clinical trials on ALN-RSV01, a siRNA-based inhaled treatment for respiratory syncytial virus (RSV) that targets the nucleocapsid encoding gene in the virus. (Alvarez, et al., 2009) Lung transplant patients exhibited improved symptom scores and overall lung function. Alnylam has also developed ALN-VSP, a cocktail for two siRNAs that target vascular endothelial growth factor and kinesin spindle protein, as a systemically delivered liver cancer therapy. In Phase I trials, ALN-VSP was well tolerated and reduced tumor blood flow in patients. Finally, Alnylam has initiated a Phase I study of ALN-TTR01, a siRNA therapeutic that targets transthyretin (TTR), a carrier for thyroid hormones and retinol binding proteins, which is mutated in hereditary TTR-mediated amyloidosis. Pre-clinical trials have demonstrated that ALN-TTR01 can cause regression of amyloid deposits and silence the TTR gene.

Other companies have multiple RNAi-based drugs in the R&D pre-clinical and Phase I pipelines, targeting a wide range of disease conditions, including age-related macular degeneration (AMD) and various cancers. (Vaishnaw, et al., 2010) In addition to the Alnylam siRNA drug against RSV, there have been some inroads into development of RNAi-based therapies to treat infectious disease. For example, Tacere Therapeutics has developed a RNAi-based cocktail that targets three separate conserved regions of the Hepatitis C virus (HCV) and can be delivered to liver cells via intravenous administration using encapsidation in an adeno-associated protein coat. In animal studies, this therapeutic agent targeted and cleaved HCV at three different sites simultaneously without toxicity. In a

pilot Phase I/II clinical trial, Benitec Ltd., in collaboration with City of Hope National Medical Center, demonstrated long-term expression of three RNA-based anti-HIV moieties (tat/rev short hairpin RNA, TAR decoy, and CCR5 ribozyme) in hematopoietic progenitor cells that support the development of an RNA-based cell therapy platform for HIV. (DiGiusto, et al., 2010) The gene-modified stem cells had been infused into HIV-positive patients via autologous bone marrow transplantation to treat AIDS-related lymphomas. In another study, intranasal delivery of siRNAs in a SARS coronavirus (SCV) rhesus macaque model was also effective in reducing SARS-like physiological symptoms, RNA expression of SCV genes, and lung histopathology associated with viral disease. (Li, et al., 2005)

Challenge	**Methods to address challenge**
RNA stability	Locked nucleic acids (LNA) Short (7-8-mer) seed-directed anti-miRs
Off-target effects	
Sequence-dependent	Bioinformatic screening algorithms
Sequence-independent	Evaluation of immunostimulatory properties Reduction of RNA expression levels Use of different RNA backbone modifications DNA sequence substitution
Systemic delivery	
Chemical modifications	Cholesterol Chitosan Lipophilic molecules (e.g. bile acids)
Packaging carriers	Lipid nanoparticles (LNPs, SNALPs) Multiple lipid bi-layer nanoparticles (Atuplex) Transferrin-decorated cyclodextrin particles Nanoparticles decorated with leukocyte receptor antibody Dynamic polyconjugates

Table 1. Transition of RNAi-based approaches to the therapeutic arena

It is also informative to describe siRNA-based clinical trials that were terminated prematurely due to conclusions that the study was unlikely to yield an effective drug. Bevasiranib (Opko Health Inc.) and AGN211745 (Allergan Inc.) were developed to target the vascular endothelial growth factor (VEGF) pathways to treat patients with AMD via the intravitreal route. The overgrowth of blood vessels behind the retina causes irreversible loss of vision. Despite initial positive reports of efficacy demonstrating reduced neovascularization upon direct ocular injection of VEGF siRNA (Shen, et al., 2006), both studies were terminated during Phase II/III of clinical trials, amid suggestions that the two siRNAs activated TLR3 to mediate its effects in preclinical models, rather than direct inhibition of target gene expression. (Kleinman, et al., 2008, Vaishnaw, et al., 2010)

6.3 Delivery of stable siRNAs into the body

A major technical challenge for RNAi-based therapy is drug stability in the body and efficient systemic delivery in sufficient quantity to have a therapeutic effect. Given their size and negative charge, siRNAs cannot easily cross the host cell membrane. Unmodified siRNAs injected into the body may be subject to RNase-mediated degradation and rapid renal excretion. Thus, therapeutic siRNAs are often chemically modified and/or packaged into carriers for delivery into the host. Various means of delivery have been tested in murine and non-human primate models, including in nanoparticles, complexed with polyconjugates, attached to cholesterol groups, or conjugated with cell surface receptors. (Table 1)

Tekmira Pharmaceuticals Corp. has developed stable nucleic acid lipid nanoparticles (LNPs, formerly referred to as SNALPs), composed of several non-covalently associated components, including an ionizable lipid, polyethylene glycol (PEG)-lipid, cholesterol, and a neutral lipid. LNPs containing siRNAs that target several Zaire Ebola (ZEBOV) viral proteins were delivered into a lethal non-human primate macaque model of ZEBOV-mediated hemorrhagic fever. (Geisbert, et al., 2010) All macaques given seven post-exposure treatments were protected against ZEBOV, demonstrating the efficacy of LNP-mediated siRNA therapy for emerging viral infections. Tekmira has also applied LNP-based delivery to a Phase I trial involving 23 patients with mild hypercholesterolemia. Alnylam Pharmaceuticals has also reported systemic delivery of siRNAs that target apolipoprotein B (ApoB), encapsulated in LNPs, by intravenous injection in cynomolgus monkeys. (Zimmermann, et al., 2006) A single injection was shown to last for more than 11 days, induced significant reductions in serum cholesterol and low-density lipoprotein levels, and resulted in greater than 90% target knockdown with no detectable toxicity. Silence Therapeutics has developed an independent nanoparticle approach, the AtuPlex technology, that embeds therapeutic siRNAs into multiple lipid bi-layer structures to provide systemic delivery to specific tissues. Pfizer and Quark Pharmaceuticals are currently testing delivery of therapeutic siRNAs using the Silence technology to treat AMD and diabetic macular edema in two separate Phase II trials. Pre-clinical data has indicated that therapy decreases onset of known endpoints in AMD.

Calando Pharmaceuticals has initiated a Phase I clinical trial that utilizes receptor-mediated delivery of siRNAs encapsulated in cyclodextrin particles decorated with transferrin. Cancer cells, which often overexpress the transferrin receptor, are thus more likely to take up the particles for targeted therapeutic delivery. The trial targeted a subunit of ribonucleotide reductase, an enzyme required for DNA synthesis, to inhibit cancer and tumor growth. Tumor biopsies from melanoma patients show the presence of intracellularly localized nanoparticles. Furthermore, mRNA and protein levels for ribonucleotide reductase in tumors were decreased compared to pre-dosing tissue. (Davis, et al., 2010) Another targeted receptor-based strategy is siRNA delivery to a specific class of leukocytes involved in gut inflammation. A cyclin D1 (Cyd-1)-targeted siRNA was loaded into stabilized nanoparticles, the surfaces of which incorporated an antibody specific for a receptor expressed by the leukocytes. The targeted siRNA-containing nanoparticles down-regulated the cyclin D1 target, suppressed leukocyte proliferation, and reversed experimentally-induced colitis in mice. (Peer, et al., 2008)

Cholesterol carriers enable improved siRNA uptake in the liver, with the cholesterol easily bound by low-density lipoprotein (LDL) in serum and robust LDL uptake in the liver.

(Soutschek, et al., 2004) An siRNA targeting apolipoprotein B (apoB) has been conjugated to cholesterol in order to load siRNAs into circulating LDLs for enhanced stability and to increase receptor-mediated uptake into target hepatocytes. (Soutschek, et al., 2004, Wolfrum, et al., 2007) ApoB siRNAs have also been complexed with dynamic polyconjugates, PEG, and the liver-targeting ligand N-acetyl galactosamine to achieve site-directed delivery and potent ApoB knock-down. (Rozema, et al., 2007) Lipophilic siRNAs can also bind high-density lipoprotein (HDL) and target to tissues with HDL receptors, such as gut and brain. (Chen, et al., 2010, Wolfrum, et al., 2007) Oral delivery of glucan-encapsulated siRNA particles has been reported to target Map4k4 in gut macrophages to protect mice from LPS-induced toxicity. (Aouadi, et al., 2009) Finally, the polymer chitosan has mucoadhesive properties and has been used for intranasal delivery of siRNAs specific to a BCR/ABL-1 junction sequence, into bronchiolar epithelial cells in mice, resulting in ~40% reduction of target gene expression. (Howard, et al., 2006)

7. Conclusions: Strategies and future of miRNA therapeutic applications

Recent advances in the understanding of miRNA structure and function has enabled development of novel miRNA-based strategies for combating human infectious disease. The technologies that have already been developed for stabilization and drug delivery of siRNA-based therapeutics will no doubt accelerate transition of miRNAs into the therapeutic arena. Given that miRNAs are thought to regulate tens to hundreds of genes in the cell, caution must be taken since there may be unintended downstream consequences on cell function by seemingly small alterations in miRNA expression. A recent development that may greatly advance anti-miR therapeutics is the silencing of miRNA families with short LNA antagomirs that specifically target the miRNA seed sequences. (Obad, et al., 2011) The relatively short 7-8 nucleotide lengths of these LNA sequences may bypass the need for carrier formulation for systemic administration in the host and reduce the manufacturing costs of RNAi therapeutics. Further research, including clinical trials, will determine the efficacy of these short antagomirs for treatment of human disease.

Several companies have been established to specifically develop high-impact medicines based on miRNAs, including Santaris Pharma A/S, Mirna Therapeutics, and Regulus Therapeutics. As aforementioned, Santaris has developed a LNA-based anti-miR-122 drug, Miravirsen, to inhibit HCV infection in Phase II clinical trials. Mirna Therapeutics has demonstrated intravenous administration of a neutral lipid emulsion to facilitate systemic delivery of tumor suppressor miRNA mimics modeled after the natural tumor suppressors let-7 and miR-34 to inhibit tumor growth. (Trang, et al., 2011) Regulus Therapeutics is focusing on both miR-21 as a potential target to reverse fibrosis and cancer onset and miR-122 to reduce cholesterol levels and inhibit HCV infection. Other studies in the laboratory have implicated miRNAs in key organ function. For example, the cardiac-specific miR-208 is required for cardiomyocyte hypertrophy, fibrosis and expression of bMHC in response to stress and hypothyroidism. (van Rooij, et al., 2007) This momentum in development of RNAi-based drug strategies represents an exciting time for translational research that links fundamental bioscience discovery in cancer and infectious disease to therapeutic treatment. The overall promise of miRNAs as a powerful new approach to induce sequence-specific inhibition of gene expression has generated enormous enthusiasm and hope in the biomedical community that miRNA-based therapeutic treatment of disease can become a reality in the near future.

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9. References

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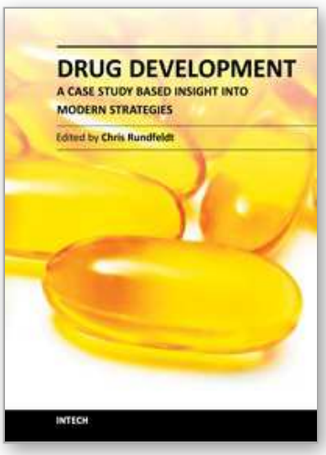
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This book represents a case study based overview of many different aspects of drug development, ranging from target identification and characterization to chemical optimization for efficacy and safety, as well as bioproduction of natural products utilizing for example lichen. In the last section, special aspects of the formal drug development process are discussed. Since drug development is a highly complex multidisciplinary process, case studies are an excellent tool to obtain insight in this field. While each chapter gives specific insight and may be read as an independent source of information, the whole book represents a unique collection of different facets giving insight in the complexity of drug development.

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