Targeted therapies through microRNAs: pulp or fiction?

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Abstract: With the discovery of post-transcriptional silencing, the idea of targeted therapies became an approachable goal. The widespread involvement of microRNAs (miRNAs) was thought to be the magic bullet against multiple diseases. However, several hurdles, ranging from targeted delivery to side effects still have to be resolved. In this review, we discuss recent progress on delivery as well as current applications for miRNAs as therapeutic agents.

Keywords: microRNA, delivery, post-transcriptional gene silencing, cancer

Introduction

Throughout the last decade, the promise of targeted, personalized cancer therapies has triggered a growing number of clinical trials, especially with monoclonal antibodies and receptor tyrosine kinases inhibitors. Next-generation sequencing, the use of microarray technology, high-throughput small hairpin RNA screens as well as improved animal models have led to the identification of disease-specific mutations and aberrantly expressed genes. Despite the clinical success stories of small molecule inhibitors and monoclonal antibodies such as imatinib and rituximab, the therapeutic backbone of most tumor therapies still remains conventional chemotherapy.

Mechanisms of gene silencing

With the recent discovery of RNA interference (RNAi), specific gene-knockdown tools became available to the scientific community and were soon developed towards a more clinical application. In general, a gene-specific knockdown can be achieved in mammals through the application of antisense RNAs, endogenous microRNAs (miRNAs), and exogenous small inhibiting RNAs (siRNAs) (Figure 1). siRNAs as well as miRNAs act through RNAi, leading to mRNA decay and translational inhibition [Carthew and Sontheimer, 2009].

RNAi is a conserved mechanism of post-transcriptional gene silencing, mainly exerted by

miRNAs in mammals [Lagos-Quintana *et al.* 2003; Martinez *et al.* 2002]. Its catalytic core is AGO1–4 proteins, members of the highly conserved Argonaute protein family [Meister *et al.* 2004]. Based on the complementarity of an approximately 22-nt long RNA with its mRNA target, silencing can be divided into two mechanisms: direct, sequence-specific cleavage induced by siRNAs as well as translational repression exerted by miRNAs [Carthew and Sontheimer, 2009]. In contrast to siRNAs, which rarely exist in mammals, over 1000 human miRNAs have been described (www.mirbase.org).

miRNAs, cancer and cellular identity

Much evidence implicates miRNAs as contributing factors in the pathogenesis of cancers. A provocative observation was made by Calin and colleagues who found that a large number of known recurrent genomic alterations involved in cancer are in close proximity to miRNA genes [Calin *et al.* 2004]. This suggested that these rearrangements affect the expression of miRNAs with tumor suppressive or oncogenic properties. Indeed, there are numerous reports of candidate miRNA expression signatures in various neoplasias [Bloomston *et al.* 2007; Iorio *et al.* 2007; Volinia *et al.* 2006, 2010; Lu *et al.* 2005]. Translocations can result in the activation of genes involved in apoptosis or cell-cycle progression. Some of these oncogenes directly mediate the expression of miRNAs.

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Figure 1. Differences and similarities between siRNAs (left) and miRNAs (right). miRNA, microRNA; RISC, RNA-induced silencing complex; siRNA, small inhibiting RNA.

For example, overexpression of c-Myc leads directly to transcription of the miR-17-92 miRNA cluster (which, in turn, is a post-transcriptional regulator of c-Myc) [O'Donnell *et al.* 2005]. Hence, miRNA profiling revealed expression changes that are either directly causative or the result of upstream changes in the development of cancer. Depending on their targets, miRNAs can act as tumor-suppressor genes or oncogenes. Examples are miR-15a and miR-16, two miRNAs that have tumor-suppressor functions by downregulating the anti-apoptotic BCL2 protein in normal B cells [Cimmino *et al.* 2005]. BCL2 overexpression is observed in multiple B-cell malignancies, including chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphomas [Tsujimoto *et al.* 1985]. Reduction of miR-15b and/or miR-16 leads to BCL2 overexpression and it is thought to be an important mechanism in the development of B-CLL [Cimmino *et al.* 2005]. Another illustrative example of tumor-suppressive miRNAs is the repression of ras oncogenes, as well as HMGA2 by let-7, a relationship that is conserved between nematodes and humans [Mayr *et al.* 2007; Johnson *et al.* 2005]. Further studies, especially in the hematopoietic system, demonstrated a strong connection between miRNA expression and cellular identity [Lu *et al.* 2005, 2008; Petriv *et al.* 2011]. Considering the relatively small number of miRNAs encoded in the genome (www.mirbase.org), it is remarkable that miRNA

expression patterns are informative with respect to cancer diagnosis as well as the developmental origins of the tissues [Lu *et al.* 2005]. The relevance has been recently demonstrated further by using miRNAs for inducing pluripotent stem cells [Li *et al.* 2011; Saunders *et al.* 2010; Mallanna and Rizzino, 2010]. These findings demonstrate that RNAi is a powerful machine that potentially can manipulate diseased organs and tissues. Eventually, siRNA and miRNA drug development will evolve in parallel, because most of the obstacles to RNAi-based cancer therapeutics are the same for both siRNAs and miRNAs.

Delivery strategies

What are the essential properties of a drug? Depending on the architecture of a drug, common aspects include a favorable bioavailability, a reasonable half-life, and few side effects. Considerations such as the organ to be treated determine the delivery strategy. Targeting the liver meets different pharmaceutical requirements than, for example, the brain, bone marrow, or skin. So far, various strategies have been developed to overcome physiological boundaries prohibiting small RNAs from entering the target cell (Figure 2). The half-life of small RNAs in the extracellular fluids is limited by ribonucleases, renal clearance, and trapping in the liver. Then, small RNAs have to pass the endothelial

Figure 2. From injection to target knockdown. After injection into the bloodstream, siRNAs/miRNAs have to pass the endothelium and penetrate the cell membrane to become incorporated into the RISC. Several potential obstacles, as well as barriers, ranging from nucleases to off-target effects, resulting in inefficient target knockdown are shown here. ECM, extracellular matrix; miRNA, microRNA; RISC, RNA-induced silencing complex; siRNA, small inhibiting RNA.

barrier and plasma membrane of the target cell. It is worth mentioning that tumor vessels are organized so that they are more permeable than normal vasculature and allow the passage of particles as large as 200 nm compared with 5 nm in healthy endothelium (sometimes referred to as the enhanced permeability and retention effect) [Maeda *et al.* 2000].

The negative charge as well as their size inhibits siRNAs from crossing the cell membrane. Once inside the cell, siRNAs as well as miRNAs must find their way into the RNA-induced silencing complex (RISC), eventually knocking down their target(s) with as few off-target effects as possible. Delivery still remains a major challenge to be resolved. It is not clear which combination and targeting strategies should be employed, especially for metastasized cancers as well as cancers in closed compartments such as bone marrow.

A considerable amount of research has been undertaken to find the perfect delivery strategy. Every delivery strategy tested in the mammalian system, ranging from direct injection into organs and organelles to systemic approaches using nanoparticles, liposomes or aptamers, has advantages and disadvantages, and might be suitable only for specific organs and body compartments.

The half-life of a siRNA or miRNA mimic can be extended by chemical modifications involving the substitution of 2' fluoropyrimidines [Morrissey *et al.* 2005], or a 2'-O-methyl [Jackson *et al.* 2006], for the 2' ribose as well as the addition of phosphorothioate linkages [Morrissey *et al.* 2005], with little to no effect on silencing activity, but resistance to ribonucleases. Another common modification frequently used in miRNA profiling as well as miRNA antagonists is the locked nucleic acid technology [Elmen *et al.* 2005], which forms a bridge between the 2' oxygen and 4' carbon, thus bringing the ribose into a confirmation lock and allowing better hybridization properties, and currently is being used in a phase I/II trial as an antagonist of miR-122. Effective penetration of cell membranes was achieved by the introduction of liposomal formulations (extensively reviewed by Mufamadi and colleagues [Mufamadi *et al.* 2011]). These formulations are subject to diffusion or endosomal trafficking through the cell wall. Current delivery strategies with their advantages and disadvantages are summarized in Table 1. These range from chemical conjugations of RNA allowing enhanced binding to cell receptors to packaging with nanoparticles or liposomes, which can be modified for cell-specific targeting. In addition, the fusion to aptamers, mainly oligonucleic acids or peptide molecules that lead to the recognition of cell-surface receptors, paved the way for highly specific targeted therapies.

Table 1. Current delivery strategies.

Modification/ packaging	Description	Advantages	Disadvantages	References
Naked siRNA/ miRNA	Unmodified RNA	None	Rapid degradation, accumulation in kidneys, immune stimulation	McManus and Sharp, 2002
Cholesterol- conjugation	Conjugation of cholesterol to the 3' end of the sense strand	Increased stability and serum protein binding, reduced urinary excretion	No specificity	Soutschek <i>et al.</i> 2004 Lorenz <i>et al.</i> 2004
Aptamer- conjugation	Synthetic short oligonucleic acid/ peptide molecules that bind to a specific target molecule	High specificity/ affinity/stability, low immunogenicity, relatively cheap generation	Susceptibility to enzymatic degradation, expensive to generate large amounts	McNamara <i>et al.</i> 2006 Chu <i>et al.</i> 2006
Antibody- conjugation	Antibody attached siRNA	High specificity/ affinity/ stability, can be combined with delivery	Expensive to generate, high immunogenicity	Peer <i>et al.</i> 2008 Toloue and Ford, 2011
Liposomal encapsulation	Cationic lipids, PEG-lipids	High stability, efficient encapsulation and membrane diffusion	Complement activation	Yano <i>et al.</i> 2004 Santel <i>et al.</i> 2006

miRNA, microRNA; PEG, polyethylene glycol; siRNA, small inhibiting RNA.

Clinical trials

In contrast to siRNAs, miRNAs have only been used reluctantly in clinical settings. Reasons for this might be that miRNAs are able to target more than one signaling cascade, bearing potential side effects in other organs and affecting their therapeutic efficacy. On the other hand, it is not clear if the amount of delivered miRNA affects the knockdown efficacy and target selection. This ambivalent behavior has been recently shown for miR-125b, an miRNA highly expressed in hematopoietic stem cells [Petriv *et al.* 2010]. Enrichment of miR-125a or miR-125b (both have the same seed region) led to an increase of hematopoietic stem cells [Guo *et al.* 2010; Ooi *et al.* 2010]. In addition, miR-125b is able to cause leukemia when overexpressed [Enomoto *et al.* 2011; Klusmann *et al.* 2010; Bousquet *et al.* 2010], demonstrating the thin line between possible therapeutic implications and causing disease within the same miRNA family. Depending on the transcriptional background, overexpression of miR-125b in fetal liver causes predominantly acute lymphoblastic leukemia (ALL) [Bousquet *et al.* 2010], whereas in bone marrow cells, overexpression of miR-125b causes acute myeloid leukemia [O'Connell *et al.* 2010] (Figure 3). In addition, especially in fetal liver depending on the

achieved miRNA expression levels, the phenotype varies considerably between T-ALL and B-ALL [Bousquet *et al.* 2010] (Figure 4). These findings indicate that in contrast to a single-gene knock-down by an siRNA, regulation and therefore delivery of miRNAs are more complex.

Most clinical trials involving miRNAs investigate the role of miRNAs as predictive markers (<http://clinicaltrials.gov>) in cancer or inflammatory diseases. Although miRNAs have been implicated in multiple pathophysiological conditions, their use as a therapeutic target has been limited so far. Only one antagomir against miR-122 has been used in primates with hepatitis C virus (HCV) infection [Lanford *et al.* 2010]. The mechanism by which miR-122 regulates HCV replication is not based on 3'UTR binding of a predicted target gene, but in contrast on the 5'UTR binding of the miRNA [Machlin *et al.* 2011]. This activates HCV translation and a further, unidentified stage of the replication cycle. The conserved sites are located upstream of HCV internal ribosomal entry sites, allowing translation initiation [Henke *et al.* 2008]. The process seems to be RISC-dependent, but how exactly the process works is still unclear. Inhibition of miR-122 led to a substantial decrease of *in vivo* HCV replication in primates without

Figure 3. The target range of an miRNA depends on the transcriptional background. Depending on the expressed mRNAs (transcriptome A/B) within a cell, a single miRNA can have varying targets as exemplified (miRNA A). This may result in the activation/repression of different pathways. miRNA, microRNA.

Figure 4. The targets of an miRNA depend on the abundance of miRNA. Depending on the expression level of the miRNA, the number of targets might change, as exemplified (miRNA A levels). This might have consequences on the targeted pathways. miRNA, microRNA.

Table 2. Clinical studies involving microRNAs.

Study title	Disease/target	ClinicalTrials.gov Identifier
A Phase 2a, Randomized, Double-Blind, Placebo-Controlled, Ascending Multiple-Dose Study to Assess Safety, Tolerability, Pharmacokinetics and Antiviral Activity of SPC3649 (Miravirsen) Administered to Treatment-Naïve Subjects With Chronic Hepatitis C (CHC) Infection	Hepatitis C/miR-122	NCT01200420
The Predictive Role of Serum MicroRNA-122 to the Clinical Course of Chronic Hepatitis C Patients	Hepatitis C	NCT00980161
MicroRNA Expression in Renal Cell Carcinoma	Renal cell carcinoma	NCT00743054
MicroRNA Expression Profiles in High Risk Prostate Cancer	Prostate cancer	NCT01220427
MiRNA Profiling of Breast Cancer in Patients Undergoing Neoadjuvant or Adjuvant Treatment for Locally Advanced and Inflammatory Breast Cancer	Breast cancer	NCT01231386
The Expression and Significance of miRNA Profile and Markers of Inflammation in Patients With Pulmonary Arterial Hypertension	Pulmonary arterial hypertension	NCT00806312
Inflammatory Mediators and microRNA Analysis in Premenstrual Asthma	Asthma	NCT00837395
miRNA in the Evaluation of the Value of Sepsis Prognosis Prospective Observational Study	Sepsis	NCT01207531
Cytomegalovirus (CMV) microRNA Expression in Vivo and Immune Evasion Correlates	Solid organ transplants	NCT00677482
Search for Predictors of Therapeutic Response in Patients With Carcinoma of the Ovary, the Fallopian Tube or Peritoneal Serous-type Advanced Stage	Ovarian carcinoma	NCT01391351
Pilot Study of RNA as a Biomarker for Autosomal Dominant Polycystic Kidney Disease	Polycystic kidney disease	NCT01114594
Integrated Whole-Genome Analysis of Hematologic Disorders	Hematologic diseases	NCT01108159

any considerable side effects [Lanford *et al.* 2010]. These findings led to a clinical phase I/II trial, which is expected to complete enrollment by the end of 2011. All other listed clinical trials investigate the role of miRNAs as predictive markers in serum (see the recent review by Schöler and colleagues [Schöler *et al.* 2010]) or solid tissues (Table 2).

Summary

The ability of miRNAs to regulate multiple pathways as well as one pathway at various checkpoints makes them in theory attractive therapeutics. So far, only an antagomir of miR-122 for the treatment of HCV has found its way into a clinical trial. In reality, miRNAs have not yet met the magic bullet expectations for

the treatment of cancer or metabolic diseases. Reasons could be the relatively small number of animal studies using miRNAs as well as the challenge of an adequate delivery strategy. In addition, almost nothing is known about possible side effects caused by the enrichment of, for example, tissue-specific miRNAs in other organs. The field stills needs more information from innovative studies demonstrating the reprogramming of cancer cells through miRNAs, as well as targeted delivery strategies exploiting cancer-specific surface markers.

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Conflict of interest statement

The authors declare no conflicts of interest in preparing this article.

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