RNA Interference

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Tumor-targeted Delivery of Anti-microRNA for Cancer Therapy: pHLIP is Key**

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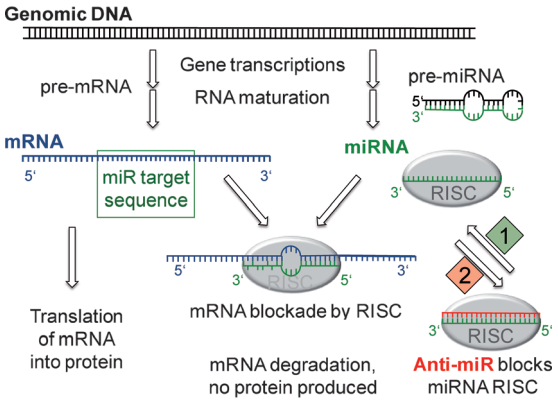
Enhanced understanding of the RNA interference (RNAi) process creates novel medical opportunities. The human genome encodes more than 1700 microRNAs (miRs), which are expressed in a tissue- and disease-dependent manner. Transcribed miR undergoes a maturation process, with a 21–23 nucleotides containing RNA guide strand incorporated together with argonaute protein into an RNA-induced silencing complex (RISC). RISCs recognize complementary messenger RNA (mRNA) strands by Watson–Crick base pairing (perfect match in the first seven or eight nucleotides at the 5′ end of the guide strand) and block the translation of mRNA into a protein (Scheme 1). Thus, the expression of genes is regulated on the posttranscriptional level.

The dysregulation of miR expression has been associated with human diseases. In cancer, the loss of tumor suppressor miRs (such as miR-34) results in more aggressive, metastatic, or chemoresistant tumor cells. The therapeutic re-introduction of suppressor miRs into tumors (Scheme 1, route 1) may reverse the malignant phenotypes. The clinical development of an miR-34 mimic (as lipid-based formulation) is ongoing for patients suffering from liver cancer or liver metastasis.[1] Conversely, other miRs, so-called onco-miRs, promote tumor growth and aggressiveness. Such onco-miRs can be inactivated by specific base pairing with antisense molecules (8–23 nucleotides), termed miR antagonists (anti-miRs, Scheme 1, route 2), thus recovering benign protein expression profiles.[2]



Scheme 1. microRNA interference. Diamonds present options for therapeutic actions (introduction of 1. synthetic pre-microRNA or miR mimics for inducing RNAi; or 2. anti-miRs such as PNA or tiny-LNA, for inhibition of RNA interference by masking RISC RNA strand).

The number of miR and related RNAi therapeutics in clinical testing is steadily increasing.[3] Intracellular delivery, ideally in a tissue-targeted manner, is the key limitation to further progress. RNA degrades in a biological environment and, as a medium-sized polyanion, cannot diffuse passively into cells. In order to solve this problem, several chemical strategies are currently being evaluated, including 1) chemical backbone modifications, 2) nanoparticle formulations, and 3) oligonucleotide conjugates. Chemical modifications have been introduced to directly stabilize oligonucleotide backbones. They include peptide nucleic acid (PNA), locked nucleic acid (LNA), or tricycloDNA chemistry.[4] To facilitate the cellular uptake, sequences were minimized with regard to the number of nucleotides, resulting in potent single stranded siRNAs or tiny LNAs. Alternatively, inspired by the delivery of natural viruses, RNAi molecules have been incorporated into virus-like nanoparticles. These utilize cell binding and other transport domains for targeted intracellular delivery. A third strategy takes advantage of both foregoing strategies: oligonucleotides with improved chemical backbones have been conjugated with small transport domains. They include receptor-targeting ligands for cell binding or cell-penetrating peptides for crossing cell-surface or endosomal membranes.[5] These conjugates may target specific sites and, as a result of their smaller size, are more likely to diffuse into target tissues compared with larger nanoparticles. For example, the subcutaneous injection of siRNA, conjugated with synthetic trimeric N-acetyl-galactosamine ligand targeted to the asialoglycoprotein receptor of liver cells, resulted in very promising gene silencing in mice[6] and men.[3] For many RNAi drug delivery approaches, the liver turned out to be the most amenable target organ. Systemic targeting of other tissues had been less successful. Debates on the most productive cell-entry mechanism (by directly crossing the

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cell membrane or through endocytosis into vesicles and subsequent release to the cytosol) are ongoing.

The recent paper by Cheng et al.[7] demonstrates the therapeutic potential of anti-miR peptide conjugates for cancer therapy. The anti-miR agents were based on PNAs that were linked with a membrane translocation peptide through a disulfide bond. These anti-miR conjugates were applied in several tumor models, including disseminated lymphoma tumors that spontaneously develop in transgenic mice (Figure 1). The work can be regarded as a masterpiece of

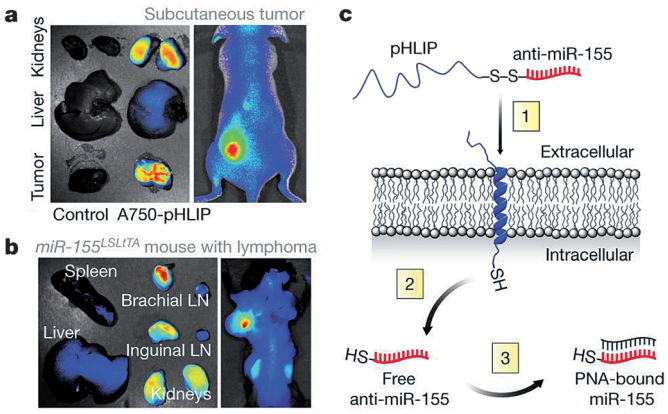

Figure 1. Targeting miR-155-addicted lymphoma using pHLIP. Targeting distribution of pHLIP labeled with Alexa Fluor 750 (A750) at 36 h after systemic administration to a) a nude mouse with miR-155 flank tumors and b) an miR-155 mouse with lymphadenopathy. Control: A750-cysteine. LN: lymph nodes. c) Schematic illustration of pHLIP-mediated PNA anti-miR-155 delivery. 1) At pH < 7, the C terminus of pHLIP inserts across lipid bilayers, which facilitates the delivery of attached anti-miR-155 PNA. 2) The disulfide between pHLIP and anti-miR-155 is reduced in the cytosol. 3) Intracellular anti-miR-155 PNA is free to bind and inhibit miR-155. Reprinted from from Ref. [7] by permission from Macmillan Publishers Ltd, Nature Publishing Group (2015).

interdisciplinary work between membrane biophysics, nucleic acid chemistry, delivery, and microRNA tumor biology. More than two decades of important discoveries were finally merged into a significant breakthrough. Uncharged polyamide PNAs were first described by Nielsen and Buchardt in 1991.[8] In the late 1990s, Engelman and co-workers demonstrated that transmembrane peptides such as the C-helix in bacteriorhodopsin can spontaneously insert into phospholipid bilayers in a pH-dependent manner. Later on, they developed a related peptide termed pHLIP, the pH (low) insertion peptide, which is a 38 amino acid long hydrophobic, negatively charged peptide. Under slightly acidic conditions (around pH 6.5), this peptide inserts as a helix into cell membranes. Cargo molecules, when attached to the C terminus of pHLIP, are translocated across the plasma membrane into living cells. When linked by disulfide bonds, the cargo molecules are released in the reducing intracellular environment (see Figure 1c). The delivery however was restricted to small, uncharged molecules. Delivery of a 20 bases long oligonucleotide was unsuccessful; in contrast, PNAs could be successfully transferred into the cytosol.[9] This direct crossing of cell membranes is notably different from the majority of delivery pathways, which commonly proceed by initial uptake into endosomes and subsequent membrane crossing.

For in vivo tumor targeting, Cheng et al.[7] took advantage of acidosis (below pH 7), which is a hallmark of solid tumors and also characteristic of a variety of other pathological conditions (such as inflammation or infection sites). Cellular uptake and functional pHLIP-PNA anti-miR delivery was demonstrated in vitro to proceed at extracellular pH 6.2, but not physiological pH 7.4. The main in vivo studies focused on onco-miR-155 and lymphoma. Overexpression of miR-155 occurs in many cancers and is able to induce the development of lymphomas.[10] Slack and co-workers developed a Tet-Off-based transgenic mouse model, in which miR-155 is induced in hematological tissue. At the age of two to three months, these mice spontaneously develop disseminated lymphoma. The authors demonstrated tumor targeting of intravenously injected fluorescence-labeled pHLIP in the subcutaneous (Figure 1a) and the spontaneous disseminated form (Figure 1b) of lymphoma. Importantly, the material did not accumulate in the liver, which is a notorious sink for nanoagents. As a positive surprise, pHLIP conjugation reduced liver accumulation of pHLIP–anti-miR over unmodified PNA by a factor of 10. They also found targeting of the kidneys (possibly as a result of the acidity of renal tubules), from where pHLIP was excreted. Toxicology in mice did not show any signs of toxicity (body weight, blood counts, liver enzymes, kidney function and morphology) of the treatment at the highest investigated dose. Two intravenous injections of a low dose (1 mg kg^{-1}) of PHLIP–anti-miR-155 resulted in significant retardation of the growth of subcutaneous lymphoma. This was not observed in control groups using a scrambled anti-miR sequence or anti-miR-155 PNA without pHLIP. At a slightly higher dose, a significant survival advantage was demonstrated over anti-miR-155 LNA. Metastatic spread of lymphoma to other organs was blocked. Remarkably, therapeutic effects were found in the clinically most relevant spontaneous disseminated lymphoma model, with a reduction of the tumor burden in the spleen, lymph nodes, and liver. pHLIP-mediated tumor targeting and anti-miR-155 PNA therapy was also effective in other tumor types, providing a bright perspective for future anti-miR conjugate medicines.

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