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Review article

Non-viral based miR delivery and recent developments

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ABSTRACT

miRNAs are promising therapeutic targets or tools for the treatment of numerous diseases, with most prominently, cancer. The inherent capacity of these short nucleic acids to regulate multiple cancer-related pathways simultaneously has prompted strong research on understanding miR functions and their potential use for therapeutic purposes. A key determinant of miR therapeutics' potential for treatment is their delivery. Viral and non-viral vectors attempt to address the major limitations associated with miR delivery, but several hurdles have been identified. Here, we present an overview on the general limitations of miR delivery, and the delivery strategies exploited to overcome them. We provide an introduction on the advantages and disadvantages of viral and non-viral vectors, and we go into detail to analyze the most prominently used non-viral systems. We provide with an update on the most recent research on this topic and we describe the mechanism and limitations of the lipid-, polymer- and inorganic material- based miR delivery systems.

1. Overview

During the recent years, microRNAs (miRs) have emerged as an attractive tool for regulating gene expression. miRs are single-stranded RNAs of ~20–22 nucleotides, and are natural endogenous products of the gene transcription process [1], similar in structure with the exogenous siRNAs. miRs, whose biogenesis has been described in detail elsewhere [2], are non-coding RNAs that utilize the cell's RNAi mechanism to target mRNAs post-transcriptionally, and downregulate their final expression [1] (Fig. 1).

The RNAi mechanism was discovered in parallel with miRs, originally in the *Caenorhabditis elegans* genome. The Argonaute and Dicer proteins have a central and prominent role to the RNAi mechanism, being at the core of the RNA Induced Silencing Complex (RISC), a complex system of proteins that performs the gene silencing, by incorporating a miR strand into the RISC complex and targeting mRNAs with complementary sequences [3,4]. To appreciate the importance and mechanistic potential of the miRs, one can simply realize that in order for the miRs to exert their action, either partial or full complementarity between the seed region of the miR and (primarily) the 3′ untranslated region (3′ UTR) of a messenger RNA is required [5,6], although additional mechanisms of interactions have been reported [7]. This allows for a single miR to have multiple mRNAs as potential targets, thus being capable to influence different pathways. Acknowledging that there have been more than 5000 miRs currently identified, with more than 3500 just recently been added to our knowledge in 2015 [7], it is not surprising that miRs arise as critical post-transcriptional regulators of gene expression, and consequently of cell proliferation, apoptosis and differentiation, among others [1]. In fact, it is estimated that the miRs regulate > 30% of the cell's genes [3].

Recurring reports indicate that the onset and progress of many diseases are associated by misexpression or dysfunction of miRs, with among such diseases being cancer biogenesis, progression and metastasis [8–11]. Profiling of miR expression between normal and cancer tissue and subsequent evaluation of the activity of miRs led to the development of two major classifications for the miRs, oncogenic or tumor suppressors [12]. As their name indicates, the former class of miRs is associated with being upregulated in tumor samples and promoting the development, growth or metastatic potential of tumors, while the latter is associate with being downregulated in tumor samples, having the capacity to inhibit tumor growth, proliferation, angiogenesis, metastasis, and inducing apoptosis or other mechanisms that impede cancer progression [12]. Indicatively, miR-155 is recognized as an oncogene, associated with tumorigenesis and metastasis and is consistently upregulated in tumors samples compared to normal tissues [13], while has demonstrated oncogenic capacity in breast [14,15], lung [16], colorectal [17], prostate [18], and skin cancers [19], among others [13,20]. In contrast, miR-34a is regarded as a master of tumor suppressor [21],

Abbreviations: EPR, enhanced permeability and retention; PEG, poly(ethylene glycol); FDA, Food and Drug administration; PLGA, poly(lactide-*co*-glycolide); PLL, poly(L-lysine); PAMAM, polyamidoamine; PPI, poly(propylene imine); DOTAP, 1, 2-Dioleoyl-3-Trimethylammonium-Propane; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; HSPC, hydrogenated soybean phospholipid; DODAP, 1,2-dioleoyl-3-dimethylammonium-propane

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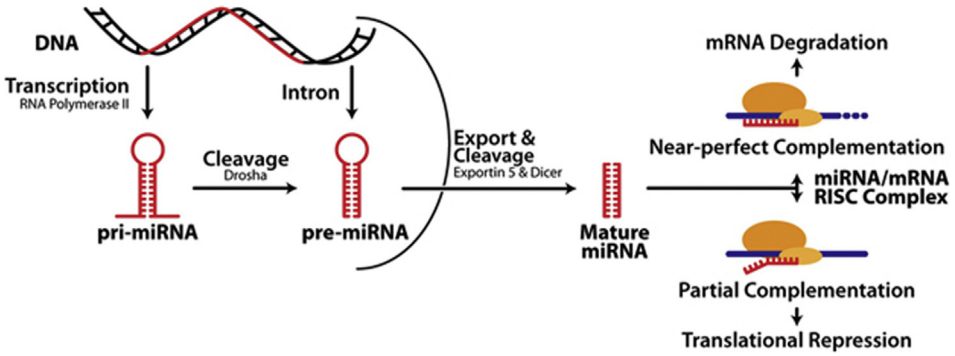

Fig. 1. Biogenesis and mode of action for miRNAs. miR biogenesis includes the transcription of the pri-miRNA from the respective gene and its cleavage by the drosha enzyme, followed by the transfer of the produced pre-miRNA by the exportin 5 proteins to the cytoplasm and the creation of the mature duplex miRNA, with the assistance of the Dicer protein.
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having potential mRNA targets in critical pathways, such as Wnt1, Notch1, Wnt3, MTA2, CD44, MYC among others [22–27]. Not surprisingly, ectopic expression of miR-34a results in reduction to cancer cell proliferation [28], migration, invasion [29], and induces apoptosis, in colon [30], lung [31], pancreatic [32], liver [33], and breast cancer [34], among others.

Understandably, miR therapeutics have become of great interest as therapeutic targets or tools in the recent years. Unfortunately, their utilization as cancer therapeutics has been impeded by inherent hurdles regarding their efficient and safe systemic delivery *in vivo*. Among the major limitations are the instability and relatively short half-life of miRs in circulation, the limited cellular uptake, instability within the endosomal compartments and limited presence in the cytosol [35].

Although several modifications of the miR structure have been studied to improve miR stability, primarily originating from prior research on siRNAs, issues with the circulation time, parenteral administration and cell uptake have not been adequately resolved [36,37]. To this end, nanovectors were the obvious next step. There have been two primary categories of nucleic acid delivery approaches, viral and non-viral vectors, both with advantages and disadvantages. Briefly, the former, as the name states, are based on genetically modified, non-pathogenic viral vectors, such as adenoviruses, capable to carry and deliver genetic material to the cells [38]. Their major limitation of the viral systems has been their immunogenic potential, creating significant side effects from the treatment, such as inflammatory responses, toxin production, even mortality [39].

In this review, we are focusing on delivery approaches based on non-viral methodologies, and we will provide an overview of current research on some of the major miR delivery systems, recognizing the existing limitations and barriers in delivering miRs.

2. Therapeutic action and delivery of miRs. Major limitations

miRs, similarly to the siRNAs, are water-soluble, making them appropriate for parenteral administration. Unfortunately, following i.v. injection, naked miRs are rapidly degraded by the abundant nucleases present in the extracellular and plasma environment, such as RNAse A-type nucleases [40]. Furthermore, the miRs tend to accumulate and be removed from the circulation to the liver and kidneys [41]. This behaviour results into a rapid drop in plasma levels within minutes post administration.

The duration of miR presence in the tissues primarily depends on whether the miRs will be uptaken by the cells and any structural modifications of the miRs that have taken place. Overall, chemical modification of the miR's structure attempts to enhance the stability, making them more resistant to degradation and hence improve their systemic presence. For example, 2′ OH group modification of the ribose ring towards 2′-*O*-methoxyethyl, 2′-*O*-methyl or 2′-fluoro modification can improve stability and binding affinity for anti-miRNA nucleic acids [42].

Wang et al. [43], administered intravenously three 2′-methoxyphosphorothioate-miRs in mice at 7.5 mg/kg, and determined that the three miRs had a plasma half-life slightly improved compared to unmodified miRs, and tissue presence, detectable up to 24 h post injection. Alternatively, Cantafio et al. [44] indicated that locked nucleic acid LNA-miR-221 inhibitor, given i.p. 25 mg/kg to mice, was detected in tumor and tissue samples up to 7 days post administration. The LNA conformation of a miR, in which a methylene bridge "locks" the ribose ring connecting the 2′-O atom with the 4′-C atom, additionally enhances the affinity and efficiency of an antagomir to inhibit the action of a target miR [45].

Among the different parameters that contribute in the duration of the presence or action of miRs in the cells include the stage of the cell cycle, the expression of growth factors or other miRs, the plurality or extend of complementary targets, and the miR itself. It is important to note that the natural miR turn-over present in the cells can further affect the levels of an exogenous miR. The reader is encouraged to read the review paper by Rugger and Grosshans [46], who present these in detail. Furthermore, there is an inherent lag between the levels of miR in a cell, the suppression of a targeted mRNA translation, the destabilization and degradation of targeted mRNA, and eventually the downregulation (reduction) at the protein level. As it was demonstrated by Bazzini et al. [47], increased levels of miR-430 do not immediately cause the downregulation of the targeted mRNA, but a lag of approximately 2 h takes place, with prior slow translational repression for the targeted mRNA. Similarly, Subtelny et al. [48], injected one-cell zebrafish embryos with miR-155 and demonstrated that there was a lag between the induced high levels of miR-155 and the destabilization of targeted mRNA. These results indicate that there will be an inherent lag between the upregulation of a miR and an observed downregulation of a specific gene.

The pharmacokinetic properties of the miRs suggest that a sufficient concentration of the miR in tumor areas will be challenging to achieve, and often result in limited presence of the miR in the tissue. Furthermore, the abnormal tumor vasculature and the higher interstitial fluid pressure in the tissue further inhibits the delivery of miRs to the targeted tumor tissue [49,50]. Finally, the tumor microenvironment has numerous cells of different types besides the malignant cells, such as macrophages, lymphocytes, adipocytes, T cells, among others, which in correlation with the complex extracellular matrix, posse an additional burden for any active compound to reach the tumor cells in sufficient quantities, including miRs [51]. The complex extracellular matrix makes it difficult for any compound to travel towards the tumor cells from the already impaired vascular system, and the non-malignant cells will uptake miRs present in the tumor area. These parameters overall will result into significantly reduced presence of the miR in the tumor environment and low availability for the tumor cells to uptake them, without potentially reaching therapeutic concentrations intracellularly. In our analysis above, we describe the case for the tumor tissue to be located in areas outside the central nervous system. In the opposite scenario, the blood brain barrier (BBB), a diffusion barrier preventing the entry of most compounds from the blood to the brain