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MicroRNA Therapeutics: the Next Magic Bullet?

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Abstract

MicroRNAs are short noncoding 18–25 nucleotide long RNA which bind and inhibit mRNA. Currently, there are over 1000 known human microRNAs, and microRNAs control over 50% of mammalian protein coding genes. MicroRNAs can be overexpressed or repressed in different diseases and inhibition or replacement of microRNAs is a promising area of study for therapeutics. Here we review the current knowledge of microRNA therapy, and discuss ways in which they can be utilized. We also discuss different methods of delivery of miRNA, and current clinical trials of microRNA-based therapies for disease. Finally we discuss the current limitations in the field, and how these limitations are being overcome.

Keywords

Antagomir; clinical trial; delivery; microRNA; microRNA mimic; therapeutic

INTRODUCTION

microRNAs (miRNAs) are highly conserved short 18–25 nucleotide noncoding RNAs which bind and post-transcriptionally regulate mRNA. The first miRNA to be discovered was lin-4 which inhibits LIN-14 in C. elegans in 1993 [1]. Since then, over 1000 mammalian miRNA have been identified, and thought to control over 50% of mammalian protein coding genes [2]. miRNAs regulate mRNA by binding to a short core sequence in the 3' untranslated region (UTR) of mRNA or non-coding RNAs [3], and lead to inhibition of translation or target mRNA degradation.

Most miRNA are processed from either miRNA DNA or introns, by RNA polymerase II into a primary-miRNA which has a hairpin structure with a 5' cap and poly(A) tail. The multiprotein complex Microprocessor (Drosha, DGCr8/Pasha) then cleaves primary-miRNA into a ~70 nt hairpin known as the pre-miRNA which is exported out of the nucleus by Exportin-5/Ran-GTP [4]. Once in the cytoplasm, Dicer (RNase III) further cleaves the pre-miRNA into a mature double stranded ~22 nt miRNA/miRNA duplex [5]. The active/guide strand of miRNA binds RNA-induced silencing complex (RISC) which compromises the

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CONFLICT OF INTEREST

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transactivation-responsive RNA-binding protein (TRBP) and Argonaute 2 (Ago2) and inhibits the 3'UTR of the target mRNA through base-pair interactions [6] (Fig. 1). miRNA can inhibit its target in one of two different mechanisms; by cleavage and degradation of the target mRNA or by repressing protein translation [6]. The mechanism that occurs is dependent on binding complementation of the miRNA to the mRNA. Perfect complementation leads to degradation of the transcript, and imperfect complementation leads to inhibition of translation [6]. Because miRNA can bind either perfectly or imperfectly it means that one miRNA can regulate many different targets allowing both specific and large scale regulation of translation. Additionally, recent studies suggest that binding of miRNA to their target sequences on other non-coding RNAs (including pseudogenes) can titrate cellular levels of miRNAs, thereby constituting another level of complexity to transcriptome regulation [3].

Due to the role of miRNA in many developmental processes, such as cell proliferation, apoptosis, development and metabolism it is not surprising that they are tightly regulated and play a role in many disease states. Due to their ability to regulate several different proteins and pathways they are also good candidates for therapeutics. This review will discuss some of the methods of replacement and inhibition of candidate miRNAs, delivery methods, and the current clinical trials involving miRNA.

Inhibition of miRNA

miRNAs are increased in many different diseases and inhibition of these miRNA would be beneficial to prevent or reverse disease progression. Many of the identified miRNA involved in disease phenotypes have been studied in cancer; for example miR-21 [7] is overexpressed in many cancers, leading to increased cell cycle and cell proliferation. In hepatitis C virus (HCV) miR-122 has been shown to play a crucial role in replication of the virus [8], and inhibiting miR-122 decreases viral load [9]. In cardiac pathological hypertrophy, miR-212/132 is overexpressed and involved in the pathological phenotype [10]. Due to the large number of miRNAs which are overexpressed in many diseases, inhibition of miRNA has become a major area of interest for gene therapy. There are several different methods that are currently being explored to inhibit miRNA binding to targets. These include; miRNA sponges, antisense antagomers and small molecule inhibitors.

Expression Vectors (miRNA Sponges)

miRNA sponges are a series of miRNA response elements placed after a reporter gene that acts as an miRNA decoy preventing binding to target mRNA [11]. miRNA sponges have been found to occur naturally as long non-coding RNA in plants and animals. For example PTENP1, a PTEN pseudogene which is mutated at the start codon so it does not produce protein [12] contains 5 different miRNA-binding elements in its 3'UTR for miRNAs that target PTEN; miR-26, -17-5p//20, -21, -19 and -214. These miRNAs are associated with regulation of the tumor suppressor gene PTEN; hence knockdown of PTENP1 in prostate cancer cells leads to an increase in these miRNAs (less is now bound to PTENP1), decreased PTEN levels and ultimately, increased cell proliferation [12].

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Synthetic miRNA sponges are usually plasmid or viral vectors which contain tandomly arrayed miRNA binding sites of between 4–10 sites, separated with a small nucleotide spacer [13] and inserted into a 3'UTR of the reporter gene driven by an RNA polymerase II promoter. Once inside the cells the sponges are amplified by the cells native RNA polymerase II. miRNA sponges can inhibit an entire family of miRNA by using the common seed sequence, and can therefore inhibit multiple miRNAs at once (Fig. 1). It has been found that miRNA binding sequences that have a 1–2 basepair mismatch in the middle of the sequence (bulge) are better at inhibiting miRNA as it slows down the RISC induced cleavage of the target sponge [14]. Studies have shown that the number of miRNA binding sites and the length of the spacer region are critical for miRNA sponge function, and some miRNA sponges have been shown to lead to long term inhibition in *in vivo* studies [15].

Another natural miRNA sponge, endogenous circular RNA, has recently been discovered. circRNAs are generated in the cell through 'back-splicing', an alternative RNA splicing [16]. Recently, circRNA7 has been shown to be regulated by miR-671 [17], and functions as an miRNA sponge for miR-7 in the mouse brain [18]. The authors also demonstrated that the testis specific *sry* circRNA acts as an miR-138 sponge, indicating that regulation of miRNA by circRNA may be common across organs. Further investigation into the structure and function of circRNA will lead to increased information on the complex area of transcriptional regulation in the cell and may help with the design of expression vectors to target miRNA.

Anti-miRNA Oligonucleotides (AMO)

Antisense oligonucleotides (AMO) have perfect complementarity to the target miRNA, bind to the guide strand and inactivate the miRNA either through inhibition of binding to the target mRNA or through degradation via recruitment of RNase H (Fig. 1). AMOs are commonly used in loss of function studies in cells and animal models and have also been the most widely used approach for therapeutic development. In order for AMOs to be effective, they need to be able to enter the target cell, be stable *in vivo* and have high binding affinities to their targets. As unmodified oligonucleotides are degraded by serum nucleases [19] and are unable to enter the negatively charged cell membrane, they need to be modified to increase stability and entrance into the cell.

Initial studies with AMOs created the first generation AMOs, also known as antagomirs. These AMOs have 2'-O-methyl modifications to increase resistance to nucleases and facilitate binding to miRNA, phosphorothioate (PS) bonds at both the 3' and 5' ends to prevent nuclease degradation, and a 3' cholesterol tail to help with cell uptake [20]. These modifications allow easier uptake of the AMO in many tissue types and can have half-lives of up to 1–4 weeks once they have reached their location. However, they have low potencies and need high dosing to be effective [21].

Second generation AMOs contain other modifications at the 2' sugar position such as 2'O-methoxyethyl which has been shown to cause better binding affinity and nuclease resistance compared with methylation [20]. 2'-fluoro, which locks sugar ring into high 3-endo conformation leads to increased affinity for the target mRNA, however these AMOs are not nuclease resistant so they still require PS linkage [20]. Locked nucleic acid (LNA)

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modifications which are bicyclic nucleic acid that tethers the 2'O to the 4'C via methylene bridge locking sugar into a 3' endo conformation have been shown to have the best binding affinity; however, due to increased binding affinity, many of these modifications can lead to off-target effects with some of the modifications being toxic. For example, as few as four LNA nucleotides in an 18mer can cause hepatotoxicity *in vivo* [22]. Also issues arise as the effect in an *in vitro* system does not always translate to an *in vivo* system, meaning each oligo needs to be tested *in vivo*. Despite these issues, the 15mer miR-122 DNA/LNA-PS AMO is safe to use in nonhuman primates and is currently undergoing clinical trials for Hepatitis C [23].

More recently, a novel compound called N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine (ZEN), when is included at each end of the AMO, led to increased binding affinity to the miRNA and inhibited exonuclease degradation. When combined with 2'O-methylation this modification looks promising *in vitro* [24]. The authors show that ZEN-AMOs have good potency and less toxicity than LNA AMOs. Another recent area of interest for therapeutics is tiny LNAs; seed-targeting 8-mer oligonucleotides which target the seed region of miRNAs. In a mouse model of lupus, anti-miR-21 tiny LNA inhibited miR-21 in B and T cells successfully altering the disease progression [25].

AMOs are the largest growing area for miRNA therapeutics, with many laboratories studying different ways to increase stability and decrease off target effects in an economical manner. The second generation miR-122 LNA was the first miRNA to enter clinical trials [23], demonstrating their therapeutic potential.

Small Molecular Inhibitors of Specific miRNAs (SMIR)

Due to the difficulty with delivery of expression vectors, antisense oligonucleotides and the cost of producing and testing AMOs, SMIR are also being pursued as potential inhibitors of miRNA function. SMIR can be designed to target miRNA at one of at least three different stages; they can interfere with primary RNA transcription, they can inhibit pre-miRNA processes by DICER and RISC, or they can inhibit the RISC and target mRNA interaction (Fig. 1). Development of high throughput screening also allows for many compounds to be tested, including ones which are already FDA approved. One caveat however is that high throughput screens for SMIRs often identify small molecules which generally target miRNA rather than specific miRNA [26], though screens to identify specific miRNA targets and their off target effects are also available.

The first specific SMIR to be discovered was an azobenzene which was shown to inhibit miR-21 by inhibiting miR-21 precursor [27]. As miR-21 plays a significant role in most cancers it is an attractive target for SMIR, and therefore, many studies have been carried out to identify compounds which inhibit this miRNA [28]. Other studies have focused on SMIRs to inhibit miR-122, which plays an important role in HCV and have identified several compounds which inhibit miR-122 [29] for further testing. SMIRs are currently limited by their relatively high EC50 and issues with specificity to a particular miRNA, however, they are much easier to deliver, and identification of SMIR which are already FDA approved, would allow for faster bench to bedside development of therapeutics.

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Replacement of miRNA

While the majority of miRNA therapeutic research has focused on miRNA inhibition, miRNA replacement therapy is another branch of miRNA therapeutics, which warrants attention due to the large number of miRNAs that are reduced in disease (Fig. 1). In most cancers, the tumor repressors let-7 [30] and miR-34 [31] are decreased, leading to increased cell proliferation. In Alzheimers disease miR-107 is decreased at early time points and therefore might be a good target for replacement [32] and in hypertrophic cardiomyopathy, miR-451 is decreased [33]. Replacement of these miRNAs by miRNA mimics could inhibit disease states through re-inhibition of their target genes and normalization of cell processes. Increasing miRNA to non-disease, basal levels in these tissues should be safe; however some kind of targeted delivery is necessary to prevent miRNA overexpression in normal tissues. As with antagomirs, most miRNA mimics need to be altered to prevent degradation by nucleases and to facilitate uptake by cells. Usually the passenger strand is altered to allow the guide strand to remain as close to the native miRNA as possible. While this area of miRNA therapy is not as well studied as AMOs, there is currently an miR-34 mimic undergoing clinical trial in patients with solid tumours, again highlighting great potential that miRNA therapies have.

Delivering microRNA

One of the largest issues with gene based therapy is the delivery of the therapeutic to the correct place without its degradation in the blood stream or excretion through the kidney. As miRNAs are small and charged they are water soluble and can be injected intravascularly or subcutaneously. However, once they are in the body they are very quickly degraded and broadly excreted via the kidney [19]. Modified miRNA as described above are much more stable and have decreased clearance; however, they are still not always accessible to the target cell.

Targeting of miRNA mimics or inhibitors to a particular tissue or cell type is an ongoing area of study. Delivery of miRNA in nanoparticles larger than 100 nm leads to quick accumulation in the liver, spleen, lung and bone marrow and non-specific uptake and excretion [34]. Furthermore even systemic administration of miRNA in cationic liposomes can lead to toxicity through toll like receptor mediated responses and immune response [35]. Some studies have utilized the properties of the delivery vector used to direct miRNA to the correct organ. For example, liposomes accumulate more readily in the lung, and therefore are good vectors for lung based miRNA [19]. AAV serotypes also preferentially enter specific cell types and can be used to target miRNA to specific organs [36]. Other studies have used targeting antibodies on the vectors to allow them to be specifically taken up by a particular cell type [37].

While the ability of miRNA to target multiple mRNA and pathways at once is a benefit, it is also a great limitation. Imperfect matching of AMO to unknown mRNA can lead to toxicity. Combination strategies of nanoparticles delivering both miRNA and siRNA to cells to silence multiple pathways at once are being explored to decrease this effect. Another issue is that RISC is often downregulated in disease states such as tumors, and without sufficient RISC, addition of antagomirs or miRNA mimics are not functionally useful.

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There are two main strategies for delivery of miRNA to the target tissue; local delivery and systemic delivery which are discussed below.

Local Delivery Methods

Local delivery of any nucleic acid has a much lower side effect profile compared to systemic delivery; however, depending on the tissue to be treated, not all targets are compatible with local delivery. Local delivery methods require the use of modified miRNAs with at least a cholesterol tail and 2'OMe to help with stability and thereby prevent degradation as well as to encourage cellular uptake. Previous studies using siRNA have shown that topical delivery of siRNA in an ethosomal carrier system is effective and has very little side effects [38]. Polyethyleneimine/miRNA complexes have also been used to locally deliver unmodified miR-145 and 33a to tumors in a mouse model of cancer [39]. Finally, nanoparticles have also been used *in vivo* for B cell cancers, with PLGA-penetratin locally injected to deliver a miR-155 antagonist to B cell tumors, preventing growth [40].

Systemic Delivery Methods

Systemic delivery methods are much more complex as the vector and miRNA have to travel through the blood stream and arrive at the correct location. Other issues such as the size of the delivery vector, the charge and the safety of the vector need to be considered when designing vectors.

Viral Vectors

Viral vectors currently have the highest efficacy for delivering miRNA into cells; however, their safety remains a controversial issue. Lentiviral, adenoviruses and adenoassociated viruses (AAV) have all been used in *in vivo* models to determine safety, efficacy and off target effects of miRNA delivery. The viral capsid can also be modified to increase affinity for specific target tissues. Lentiviruses are less safe than adenovirus or AAV as they are able to integrate their genome with the cells increasing the risk of causing cancer; however, lentiviral delivery methods have been successful with no toxicity in some models. Lentivirus delivery of miR-15a/16, which is decreased in lyphompatic leukemia, led to inhibition of disease progression in a mouse model [41]. AAV2/9 vectors have also shown promise in delivering miRNA, especially to the heart, as cardiac cells have a greater affinity for AAV9 vectors compared to other tissue types [36]. In a mouse model of dilated cardiomyopathy (DCM), AAV2/9 vectors containing miR699a, which is downregulated in DCM, led to improvement of the heart, and had stable expression for 18 months [42]. As viral delivery vectors lead to immune responses, the authors in this study injected the mice before they were 24 h old. However, AAV-based viral vectors generate less of an immune response than adenoviral vectors, and some recent studies have successfully used AAV9 mediated microRNA mimics to treat both small [43] and large animal models [44] of heart disease. Further work needs to be conducted to determine the best way to use AAV vectors to deliver miRNA in adult systems to prevent toxicity.

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Lipid Vectors

Lipid delivery vectors are lipid bilayers that contain the miRNA inside, where it is protected from degradation by nucleases, increasing their stability. They make attractive vectors as they are able to deliver their contents directly into the cell, avoiding endosome and lysosome degradation.

Cationic liposomes are beneficial because the negatively charged, hydrophilic miRNA bind directly to the lipids forming stable complexes. The positive charge also aids uptake into the negatively charged cell membrane. Cationic liposomes have been extensively studied for gene therapy delivery *in vitro* and *in vivo* [45]. Altered cationic liposomes, such as DOTMA, which can spontaneously form liposomes around its cargo have also been successful in delivering miRNA to cells, with decreased toxicity and increased delivery compared to unaltered cationic liposomes. Liposomes comprised of DOTMA and cholesterol <100 nm in size delivered miR-133b to the lungs with decreased side effects compared to the traditional NeoFX lipoplexes [46]. This was further shown with miR-29b in the lung [47].

Neutral liposomes are less toxic than cationic liposomes and do not form aggregates in biofluids, but they have decreased transfection rates *in vitro* and have less loading capacity [45]. Neutral lipids do not preferentially accumulate in the liver like cationic lipids [48], and they have higher affinity for the lung, and therefore have been utilized for delivery of miRNA to the lung. Neutral lipid emulsions have successfully been used *in vivo* to deliver miR34a and let-7 to the lung in cancer [19].

Nanoparticles

The area of nanoparticle and polymer delivery systems for gene therapy is large and growing. Several different kinds of vectors have been used *in vivo* for miRNA delivery, including altered PEG, inorganic nanoparticles, and nanoparticles with targeting abilities. For example, a Liposome-polycation-hyaluronic acid (LPH)-PEG-GC4 particle containing miR-34a mimic was successfully used to decrease lung tumor load in a mouse model of melanoma metastasis [37], while the GC4 single chain variable fragment specifically targeted tumour cells. These particles were able to deliver both miR-34a and siRNA to target genes to further decrease tumor load in these mice. These particles also did not induce proinflammatory cytokines.

Other studies have investigated the use of inorganic nanoparticles for delivery. Iron magnetic nanoparticles successfully delivered miR-16 to gastric cancer cells to inhibit Adriamycin drug resistance [49] and *in vitro*, gold nanoparticles were able to successfully deliver miR-145, which is downregulated in many cancers, to prostate and breast cancer cell lines [50]. Using magnetic particles also allows for magnetically driven targeting of disease tissue [51].

Recently, a cationic solid lipid nanoparticle containing both miR-34a and the cancer drug paclitaxel was used to inhibit tumor growth in the lungs of mice with B16F10 tumors. This demonstrates the potential for dual therapeutic regimens in both miRNA and drug leading to increased tumor death [52].

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Current Clinical Trials

Currently, there are two miRNAs that are in clinical trials; miR122 LNA to target hepatitis C virus (HCV) has currently finished phase IIb testing, and miR34, which is currently in phase I testing.

miR-122 LNA AMO for HCV

miR-122 is a liver specific miRNA, which plays an important role in facilitation of replication of the hepatitis C virus [8]. This was shown to be through interactions with two miR-122 binding sites at the 5' noncoding region of the virus [53]. Current therapies for HCV are PEG-IFN alpha and ribavirin, which have a poor side effect profile and are often ineffective. Satnaris Pharma has developed a LNA-miR-122, known as Miravirsen, which binds to the 5' end of miR-122 and decreases HCV in nonhuman primates with no side effects [9]. In Phase II clinical trials of Miravirsen, where patients with chronic HCV were given different doses of Miravirsen 5 times a week for 29 days and followed up to 18 weeks later, it was observed that in patients with chronic HCV there was a significant, dose dependent decrease in HCV, which was sustained after administration [54]. This trial demonstrated the safety and effectiveness of the therapy and is an important first step for the development of miRNA therapeutics.

miR-34 for Solid Cancers

The human miR-34 family contains three miRNAs; miR-34a, miR-34b and miR-34c. These miRNA have identical seed regions and therefore, control a similar set of target mRNAs [55]. miR-34 is transcriptionally induced by p53 [56] and has numerous targets in many different pathways. miR-34 is decreased in many types of cancer, leading to tumurogenesis [31]. Many mouse models have shown that increased miR-34 leads to decreased tumur size, with no side effects [57], however, delivery of the miR proved to be difficult. Mirna Therapuetics developed a delivery technology called NOV340, or SMARTICLES®, a liposome which forms particles of ~120 nm and are cationic at low pH and neutral or anionic at neutral or higher pH. SMARTICLEs® have efficient delivery to the liver and decreased tumour burden in mice with liver cancer [58]. Recently, MRX34, miR-34 in a SMARTICLE® liposomal injection, has entered phase I clinical trial for liver cancer and metastasis from other cancers (NCT01829971). The phase I trial will be completed in early 2015.

Current Considerations of miRNA as Therapeutics

The growth of the miRNA field over the past 20 years has been astronomical, with identification of hundreds of different miRNAs and their targets. The field though is still relatively new, and there is much to be discovered and finessed before miRNA therapy becomes a wide reaching reality. For example, the ability of miRNA to inhibit many different mRNAs and pathways at the same time may be useful for miRNAs where all targets are known, but as miRNA appear to be cell-type specific, understanding how they function in other cell types will be critical to determine specificity and off target effects. Also the ability to specifically target miRNA to a particular cell type will be imperative to prevent inhibition or overexpression of miRNA occurring in surrounding normal tissues.

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Determining the stability of the therapeutic and the length of its action in the cell will also be important when dealing with acute vs. chronic disease. Although there are still challenges to develop safe and effective miRNA therapeutics, the large amount of research occurring in this field will continue to improve development leading to more miRNA clinical trials.

CONCLUSION

miRNA have been shown to play a crucial role in cell homeostasis and when deregulated are involved in many disease phenotypes. Because of their relatively small size and the network of proteins they regulate, they are promising targets for therapeutics. Despite the problems associated with gene therapy; namely stability, delivery, and toxicity, much progress has been made in a relatively short time to develop miRNA and vectors that are suitable for therapeutic use. The advent of two miRNA to clinical trials is also promising, and as the field continues to grow and resolve stability and targeting issues, miRNA gene therapy may become a new class of drugs for a wide range of different diseases.

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LIST OF ABBREVIATIONS

AAV	Adenoassociated virus
Ago2	Argonuate 2
AMO	Anti-miRNA oligonucleotide
circRNA	Circular RNA
Hepatitis C virus	HCV
LNA	Locked nucleic acid
miRNA	microRNA
PS	Phosphorothioate
RISC	RNA-induced silencing complex
SMIR	Small molecular inhibitors of specific miRNAs
TRBP	Transactivation-responsive RNA-binding protein
UTR	3’untranslated region
ZEN	N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine

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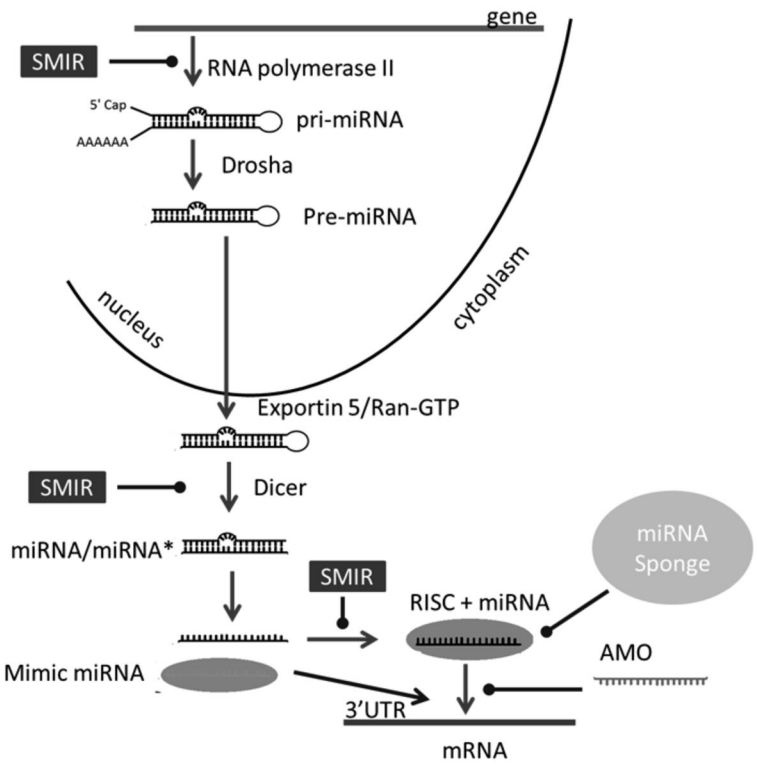

Fig. 1. The biogenesis of microRNA (miRNA)

miRNA are produced from their gene or an intron by RNA polymerase II into a ~ 70 nt long pri-miRNA which is cut by Drosha into a pre-miRNA. The pre-miRNA is transported into the cytoplasm by Exportin 5/Ran-GTP and cut by Dicer to the miRNA/miRNA* duplex. The lead strand is loaded onto RISC and then inhibits its target mRNA at the 3'UTR. To inhibit miRNA function, small molecular inhibitors of specific miRNAs (SMIR) inhibit miRNA production by inhibiting RNA pol II, Dicer, or RISC. Anti-miR oligonucleotides and miRNA sponges inhibit miRNA by binding to and inhibiting their function. To overexpress miRNA, mimic miRNA are introduced and bind and inhibit mRNA at their 3'UTR.