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## **Therapeutic modulation of microRNA for the treatment of pro-inflammatory disorders.**

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## **ABSTRACT**

microRNAs are short, non-protein coding RNAs that regulate target gene expression principally by causing translational repression and/or mRNA degradation. miRNAs are involved in most mammalian biological processes and have pivotal roles in controlling the expression of factors involved in basal and stimulus-induced signalling pathways. Considering their central role in the regulation of gene expression, miRNAs represent therapeutic drug targets. Here we describe how miRNAs are involved in the regulation of aspects of innate immunity and inflammation, what happens when this goes awry such as in the chronic inflammatory lung diseases cystic fibrosis and asthma, and discuss the current state-of-the-art of miRNA-targeted therapeutics.

## 1. MiRNA biogenesis and function

MicroRNAs (miRNAs) are a class of non-coding endogenous nucleotide RNAs that have emerged in recent years as regulators of gene expression. These highly conserved 20-25-mer RNAs regulate messenger RNA (mRNA) at the post-transcriptional level by binding principally to 3' untranslated regions (3'UTR). Formerly thought to repress the translation of target mRNA, it has been recently shown that the main function in mammalian systems is to decrease target mRNA levels [1\*].

Human miRNAs are present in introns and exons of non-coding transcripts as well as introns of coding genes (Figure 1). Primary miRNAs (pri-miRNAs) initially are transcribed from long primary transcripts by RNA polymerase II [2]. They are then cleaved by the Drosha-DGCR8 (DiGeorge syndrome critical region 8 homologue) into precursor miRNAs (pre-miRNAs) after going through a series of endonucleolytic steps [3]. Pre-miRNAs are ~70 nucleotides (nt) in length and typically have a stem-loop hairpin structure; these are transported into the cytoplasm by the RanGTP-dependent dsRNA-binding protein Exportin 5 [4]. Pre-miRNAs are further processed by the cytoplasmic RNAase III enzyme (or Dicer) into double-stranded miRNA duplexes of ~22nt in length consisting of a mature miRNA and a miRNA\* strand [5]. The former strand is incorporated into a miRNA-induced silencing complex (miRISC) which is facilitated by Argonaute protein and transported by Importin 8 to its cognate mRNA, leading to either target degradation or repression [6]. Within each miRNA there exists a 2-8 nucleotide 'seed region' which is critical for target selection, binding imperfectly yet selectively to miRNA recognition elements (MRE) within the 3'UTR of target mRNAs. However, recent studies have suggested several exceptions [7]. For example, miRNA-369-3 has been shown to increase rather than decrease the expression of TNF- $\alpha$  by binding within the latter's 3'UTR mRNA [8].

miRNAs themselves are subjected to regulatory processes. These include induction of miRNA expression by transcription factors in response to inflammatory stimuli or cellular

stresses, impaired processing due to Dicer inhibition, post-transcriptional modifications and miRNA localization to stress granules and p-bodies [9, 10]. Like other gene regulators, miRNAs display spatial and temporal effects which are crucial in the regulation of many genes involved in a variety of biological processes.

## **2. miRNAs, Innate Immunity & Toll-like receptor signalling**

The innate immune system is a complex, highly organised system which provides the first-line defence upon exposure to invading pathogens or allergens. Amongst its major functions are the recruitment of immune cells to the site of damage, induction of antimicrobial defences and priming of the adaptive immune system. Innate immunity is predominantly mediated via myeloid cells [11] however within specific organs other cell types can also play a large part. These cells commonly express pattern recognition receptors which can recognise and discriminate structures present in microbial and/or damaged-self molecules. For example epithelial cells in the lung express Toll-like receptors (TLRs) [12] which can become activated in response to microbial products such as LPS or lipopeptides or host-derived factors such as HMGB1, leading to activation of transcription factors such as nuclear factor  $\kappa$ B (NF $\kappa$ B) and the interferon-regulatory factors (IRF) [13]. miRNA modulation of innate immune mechanisms is an important fundamental process. At the most basic level miRNAs have an important role in haematopoiesis and in the establishment and maintenance of the cellular fate of immune cells [14-16\*\*]. Additionally multiple miRNAs are involved in regulating components of innate immune signalling pathways and furthermore their expression can be induced in response to various proinflammatory stimuli.

MiR-155 is an excellent example of a miRNA that participates in multiple aspects of innate immunity. It is a ubiquitously expressed miRNA [17]. Known targets of miR-155 include the transcription factor PU.1 (POU domain class 2 associating factor 1), a key mediator of monocyte and macrophage differentiation [18]; SOCS1 (suppressor of cytokine signalling 1) [19]; the inositol phosphatase SHIP1 [20]; FOXP3 (Forkhead box P3) [21], and;

MyD88 (myeloid differentiation factor 88) [22, 23]. Other targets include FADD (Fas-associated death domain protein), IKK $\epsilon$  (I $\kappa$ B kinase  $\epsilon$ ) and the TNF receptor superfamily-interacting serine threonine kinase Ripk1 [24]. The expression of miR-155 is strongly induced by inflammatory cytokines such as interferon- $\beta$ ,  $\gamma$  and also by TLR ligands through MyD88- or TRIF-dependent pathways [22, 24 25\*].

miR-146a is another miRNA identified to have an important role in the innate immune system. miR146a is mainly regulated by bacterial products and has been shown to be suppressed by LPS-treatment in a murine macrophage cell line. Directly targeting the 3'UTR of IRAK1 (interleukin-1 receptor-associated kinase) and TRAF6 (TNF receptor associated factor 6), this miRNA may play a role in turning off the LPS pathway in the absence of microbial infection, while its down-regulation may be needed for LPS-induced inflammatory response [26\*\*]. In human alveolar cells increased expression of miR-146a and b is induced by IL-1 $\beta$ , an event corresponding to a decrease in the expression of interleukin-8 and RANTES (regulated upon activation, normal T-cell expressed and secreted) [26].

In the context of TLR and IL-1R signalling the roles of a selection of miRNAs have been elucidated (Figure 2). For example members of the let-7 family and miR-223 are key miRNAs that directly target TLR4 [27, 28]. Transfection of anti-sense miRNA to let-7e in macrophages increases LPS-induced cytokine responses [29]. Meanwhile, down regulation of let-7i increases TLR4 in human cholangiocytes following LPS treatment [27]. The aforementioned miR-155 targets MyD88 whilst, miR-146a negatively regulates the expression of IRAK1 and TRAF6 [30]. The I $\kappa$ B kinases (IKKs)  $\alpha$  and  $\beta$  were recently shown to be targeted by miR-223 and -199, respectively [31, 32] and miR-9 could also directly target the NF $\kappa$ B1 gene [33].

miRNAs that target cytokines induced by TLRs have also been identified. For example, let-7 and miR-106, respectively target IL-6 and IL-10 mRNA [34, 35]. Interestingly, miRNA-induced stabilization of TNF and IL-10 mRNA has also been demonstrated for miR-369-3 and miR-446l [24, 36].

### **3. Cystic Fibrosis and Asthma: The role of miRNAs in the innate immune response of inflammatory lung diseases**

By translating these generic observations regarding the roles of miRNAs in innate immunity to clinical scenarios, the concept of miRNAs as therapeutic drug targets emerges clearly. Cystic fibrosis (CF) is a recessive genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. CF exhibits a multitude of clinical manifestations however the lung disease is responsible for the major morbidity and mortality in CF sufferers; this is characterised by defective epithelial chloride ion conductance, a decreased airway surface liquid volume, increased expression and activity of host proteases, impaired antimicrobial defences, mucus hypersecretion and a propensity to become colonised with microbes that can form biofilms within the lung [37-39]. Colonisation with *Pseudomonas aeruginosa* is associated with intrapulmonary LPS and elevated expression of IL-1 $\beta$  impacting on TLR4 and IL-1R1, respectively. Proteases such as neutrophil elastase induce expression of pro-inflammatory cytokines such as IL-8 via TLR(s), MyD88, IRAK and TRAF6. These signalling intermediates therefore represent potential therapeutic targets for miRNA-based CF therapy.

Indeed evidence that miRNAs represent modulators of the innate immune system in CF is emerging [40]. Target of Myb1 (TOM1), a negative regulator of IL-1 $\beta$ - and TNF- $\alpha$ -induced signalling pathways, is a target for miR-126. This miRNA is highly expressed in the non-CF lung but suppressed *in vivo* in the CF lung [41\*\*]. Following stimulation with LPS or IL-1 $\beta$ , over expression of TOM1 was found to down regulate NF $\kappa$ B activity whilst TOM1 knockdown resulted in a significant increase in NF $\kappa$ B-regulated IL-8 production. Over expression of miR-155 in CF has also been implicated in promoting inflammation by directly targeting and reducing SHIP1 expression and promoting PI3K/Akt activation. This caused downstream activation of mitogen-activated protein kinases (MAPKs) and concomitant stabilisation of IL-8 mRNA [42].

Asthma is another common chronic airway disease affecting approximately 300 million people worldwide. Its pathogenesis is attributed to both genetic and environmental factors leading to reversible bronchoconstriction as a result from chronic airway inflammation, mucus hypersecretion and airway hyperresponsiveness to inhaled stimuli. An abnormal T helper-2 response is usually associated with this condition and Th2-related cytokines such as IL-4, IL-5, IL-6, and IL-13 have all been implicated in asthma [43]. Studies on animal and human models have implicated miRNAs in asthma pathogenesis. miR-21, for example is highly expressed in IL-13 transgenic mice [44] and has also been implicated as a major regulator of Th1 versus Th2 responses and IFN- $\gamma$  signaling [45]. Another study showed that let-7 inhibits IL-13 expression, identifying it as a major potential regulator of Th2 inflammation [46\*]. New data on miRNAs including, once again miR-146a [47], strongly support the concept that miRNAs represent valid drug targets for the treatment of asthma (Figure 3).

#### **4. Pharmaceutical strategies to modulate miRNAs**

miRNA therapeutics are the most recent of a range of RNA therapies that have emerged over the last 10-15 years including short interfering RNA (siRNA) and short hairpin RNA (shRNA) based on manipulation of the diseased cells' RNAi machinery (Table 1).

***miRNA inhibitors:*** miRNAs that are over-expressed or have a gain of function in diseased tissue may be therapeutically targeted in a number of ways (Figure 4a). Each of the approaches aims to target endogenous miRNAs of interest through sequence complementarity and subsequently block its processing by the RISC or alternatively lead to its degradation. Anti-miRNA oligonucleotides (anti-miRs) have been developed to anneal to miRNAs and subsequently inhibit their function. Many modifications to anti-miRs exist which confer increased serum stability and higher affinity and specificity towards small RNAs [48,

49]. More advanced modifications include locked nucleic acids (LNAs) [50] and cholesterol-conjugated anti-miRs termed 'antagomiRs' [51].

**miRNA mimics:** Conversely, miRNAs that are down regulated in disease may be replaced transiently by using miRNA mimics, or more stably using a transgene approach to deliver DNA encoding primary, pre- or mature miRNA [52] generally via plasmid DNA (pDNA) (Figure 4b). Although most of the work with miRNA mimetics to date has been done with respect to cancer treatment, efforts to use these strategies in other settings are underway.

The translation of miRNA modulation into therapeutics *in vivo* will be dependent on delivery to the desired target cells. As miRNA modulation is mediated by large, anionic DNA- and RNA-based constructs many anatomical and cellular barriers to their delivery exist *in vivo* including degradation by serum nucleases, clearance by glomerular filtration, limited extravasation, poor cell membrane permeability and limited endolysosomal escape. While localised delivery to the target tissue overcomes some of these barriers, e.g. aerosolisation for delivery of miRNA therapeutics targeting respiratory conditions, the barriers to delivery are still not trivial [53]. At a cellular level, small RNAs utilise the RNA machinery, they must be delivered to the cytoplasm where the RISC is active whereas for pDNA-based strategies access to the nucleus is required for efficacy. Many delivery systems are currently under examination for use *in vivo*, and categorised into viral and non-viral approaches.

#### *Viral Delivery*

Stable expression of anti-miRs or mature miRNA may be achieved by using a viral vector such as Adenovirus, Lentivirus, and Adeno-associated virus. For the therapeutic targeting of disease associated with over-expressed miRNAs, 'miRNA sponges' are an exciting option. With the ability to be stably expressed these are transcripts containing numerous tandem-binding sites targeting a miRNA of interest, allowing for a greater number of target miRNAs to be inhibited than by using synthetic anti-miRs [54]. Conversely, miRNAs

that are down-regulated in disease (e.g. miR-126 in CF bronchial epithelium) may be replaced transiently by using miRNA mimics, or more stably using the transgene approach to deliver DNA encoding primary, pre or mature miRNA. Driven by RNA polymerase (Pol) III promoters, miRNAs can be expressed as pre-miRNAs or as artificial shRNA, the latter with the ability to circumvent Dicer processing. Although high stable expression of these transcripts can be achieved using Pol III, a major drawback is risk of severe toxicity due to the saturation of the exportin-5 pathway used by endogenous miRNA [55]. This saturation can have fatal consequences [56\*]. Perhaps a better method, with the possibility of induced ectopic or tissue specific expression of the miRNA of interest is the use of an entire primary miRNA driven by the Pol II promoter. Successfully used to over-express various miRNAs, a pri-miR-Pol II transgene system has also appeared to be of use in the expression of multiple miRNAs from a single transcript [57-59].

#### *Non-viral delivery*

Viral delivery of RNA and DNA in vivo has been associated with induction of toxic immune responses, random integration into the host genome and potential saturation of RISC machinery [60]. Therefore non-viral delivery approaches have been explored. Possibly the most advanced miRNA therapeutic currently is a 'naked' LNA against liver expressed miR-122 [61\*\*]. Constituting over 70% of the total miRNAs in the liver, this miRNA appears to be required for the replication of hepatitis C virus (HCV) [62]. Santaris Pharma has recently entered this LNA miravirsen (SPC-3649) into phase 2(a) clinical trials.

Non-viral carriers can offer improved stability and targeting over naked nucleic acids, especially for systemic delivery of miRNA therapeutics. The majority of non-viral RNA- and DNA carriers are lipid or polymer based. Cationic lipids or liposomes bind electrostatically to anionic small RNAs to form lipoplexes. Although most of the research into lipid based systems has been carried out using siRNA, evidence for their experimental use in a miRNA setting is accruing, such as with anti-miRNA ASOs [63]. Major challenges to the use of lipid

based carrier systems *in vivo* include toxicity, and activation of interferon responses and induction of inflammatory cytokines [64].

Cationic polymers are under investigation for use in miRNA delivery as they are easily manipulated chemically [65]. Cationic polymers bind negatively charged RNAs through electrostatic interactions to form polyplexes. Synthetic cationic polymers such as polyethylinimine (PEI) have high cationic charge densities, and have been used for DNA and siRNA applications for some time. However as toxicity and lack of biodegradability is an issue with PEI [53], the spotlight is turning towards the use of PEI derivatives and other biocompatible, biodegradable polymers such as chitosan which is already showing promise with siRNA based therapeutics [66]. Frequently used for targeting of therapeutic macromolecules, cell penetrating peptides have more recently been covalently or electrostatically bonded to siRNA and anti-miRNA antisense oligonucleotides with moderately successful outcomes; however more work is needed to optimise these delivery systems [67-69].

A major factor in delaying the application of miRNA therapies to the clinic is delivery and the key to its success will involve balancing between transfection efficiency and toxicity. However, many significant improvements in delivery technology have been made in recent times and show promise for the development of safe and effective miRNA based therapeutics in the future.

## **5. EXPERT COMMENTARY**

The enthusiasm with which researchers have embraced miRNA biology and the race to develop effective miRNA-based therapies over recent years has been astounding. With this change in research practice has come a measurable shift in the focus of early drug development from siRNA-based approaches to miRNA-targetted strategies. Although these two approaches are not always interchangeable, where siRNA versus miRNA-based

therapies can be developed there would now appear to be a near equal investment of time and effort in both.

With each comes challenges, however according to Aimee Jackson, a pioneer in the field of RNAi, the idea of using miRNAs rather than siRNAs to fight disease may have broader and more moderate effects [70]. In addition some aspects of miRNA-based therapies may be simpler than siRNA therapeutics. For siRNA the targets are mRNA transcripts that can be up to several thousand nucleotides in length and part of populations that encompass several splice variants: for miRNA therapies the goal is to either knock down or replace a particular natural miRNA that is fewer than two dozen nucleotides long. However notwithstanding these pros, there are downsides to take into consideration also. miRNAs rarely, if ever, target only a single mRNA transcript. Thus inhibiting or enhancing the expression of a miRNA can have off-target effects. One potential solution to this problem could be to target combinations of miRNAs that represent a 'miRNA signature' for a given mRNA. Work in our group focuses on identifying the complete miRNA-ome of individual mRNAs and using a translational research approach to determine whether these miRNA signatures are altered *in vivo*.

With respect to the treatment of respiratory disease, RNA-based therapies are particularly attractive as organ-specific targeting of RNA-therapeutics can be achieved via inhalation. Respiratory drug delivery decreases systemic exposure of the patient to the therapy, thereby reducing off-target effects. Indeed, inhaled siRNA has been one of the first RNA-based therapies to reach the clinic with Alnylam's anti-RSV siRNA treatment now reaching Phase III. It seems inevitable that miRNA-targeted strategies to treat chronic inflammatory lung diseases will follow a similar route in the coming years.

## **6. Five-year view**

Within the next five years we are likely to see continued investment of time and effort by researchers and pharmaceutical companies in the development of miRNA-targeted

therapies to treat chronic inflammatory disorders. Although organ-specific delivery systems for modulation of miRNAs *in vivo* are still in development, major advances in this area are inevitable. In our group we have been examining the feasibility of delivering pre-miRs into CF bronchial epithelial cells and our efforts have yielded very promising results to date. Using patented encapsulation technologies that can penetrate mucus, facilitate rapid and efficient cell uptake and minimise toxicity we are developing bioresponsive, inhalable hydrogels that will release miR-targetted nucleic acids directly to sites of inflammation in the lung. These customised drugs will selectively inhibit only abnormal inflammatory processes and mucus hypersecretion leaving intact the normal processes required for normal lung physiology.

In order to develop these and similar drugs effectively, clearly we also need a better understanding of the *in vivo* pharmacokinetics of viral and non-viral miRNA mimics and inhibitors. For this preclinical animal models will prove useful. Finally whilst we have made significant advances in our basic understanding of the role of miRNAs in cellular physiology, cancer and more recently inflammatory diseases, there will be further intensive research in these areas over the coming years. A particularly exciting aspect of this will be the prospect of developing personalised medicines to treat idiosyncratic disease manifestations.

## **7. Key issues**

- Anecdotally miRNAs are thought to regulate 30% of the human genome – this figure is likely to be a large underestimation with miRNAs actually playing key roles in almost all biological processes.
- In the context of innate immunity miRNAs have roles in regulating immune cell lineage development and maintenance, and in the control of individual genes involved in innate immune signalling pathways. miRNAs can be rapidly induced in response to proinflammatory stimuli to enhance the immune response.

- miRNA expression can be altered in a tissue-specific manner in individuals suffering from chronic inflammatory diseases such as cystic fibrosis or asthma. Key miRNAs that are dysregulated in this way represent new therapeutic targets for drug development.
- Strategies to over express or inhibit miRNAs exist but are still in their infancy. Currently the most significant barriers to miRNA-based medicines are the development of effective pharmaceutical strategies for targeted delivery to specific sites and with acceptably low toxicity.
- One of the most advanced miRNA-based therapies is from Santaris Pharma and has recently reached phase 2(a) clinical trials. This therapy is a locked nucleic acid (SPC-3649) targeting miR-122 in the liver for the treatment of hepatitis C virus.

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## **Figure legends**

### **Figure 1**

Primary miRNA transcripts are transcribed by RNA polymerase II and form variable-length primary transcripts (pri-miRNAs) that are subsequently converted into pre-miRNAs by RNAase III Drosha and DGCR8. These hairpin structures are transferred to the cytoplasm via the exportin transfer system (Exportin 5). The RNAase Dicer cleaves the pre-miRNA into an miRNA duplex in which the mature miRNA guide strand is incorporated into a miRNA-induced silencing complex (miRISC) while the miRNA passenger strand\* is degraded. Incorporation of the mature miRNA into miRISC is facilitated by Argonaute proteins leading to binding to the 3'UTR mRNA causing either direct cleavage, cleavage-independent mRNA degradation or direct/indirect blockade of protein translation.

### **Figure 2**

Multiple Toll-like receptors (TLR) such as TLR 2 and 4 and IL-1 receptor signal via the MyD88/IRAK/TRAF6 pathway to propagate NF- $\kappa$ B- dependent gene transcription which leads to pro-inflammatory products. Multiple miRNAs directly target components of these pathways including TLR 2, TLR 4, IL-1R, MyD88, MyD88 adapter-like protein (MAL), IL-1-associated kinase- 1 (IRAK1), TNFR-associated factor 6 (TRAF6), TAK1-binding protein 2 (TAB 2), inhibitor of NF-  $\kappa$ B kinase- $\alpha$  (IKK-  $\alpha$ ) and IKK- $\beta$  and p50 protein. The TOM1/Tollip complex and SOCS1 are examples of negative regulators of this pathway which have also been identified as targets of miRNA [22, 25, 26, 31, 32, 71, 72].

### **Figure 3**

The current understanding of miRNAs in the pathogenesis of asthma. Changes in the expression of several miRNAs are associated with development of asthma. Class 1 histocompatibility antigen G (HLA-G), an asthma susceptibility gene is a direct target of miR-

148a, b and -152 [73]. miR-146a have multiple roles including the regulation of inflammation in both human alveolar and bronchial smooth muscles cells via the B-cell lymphoma-extra large (Bcl-xL) and the IL-1 $\beta$ -induced cytokine production respectively [47, 74]. miR-21 targets IL-12p35 and pro-inflammatory tumour suppressor PDCD4 while miR-126 regulates POU domain class 2 associating factor 1 (PU.1), regulating cytokine productions such as IL-3, -5 and -10 [44, 75, 76]. Meanwhile, let-7 miRNAs inhibit IL-13 expression and thereby Th2 inflammation [46]. In ovalbumin (OVA)-induced asthma mice model, upregulation of matrix-metalloproteinase-12 (MMP-12) resulted in decreased miR-672 and 143 while miR-29 regulation of NF-kappaB (NF- $\kappa$ B)-YY1 is implicated in tissue fibrosis and remodeling [77, 78]. In another OVA-induced asthma model, the expression of RhoA is associated with downregulation of miR-133a. miR-25 and -26a directly target Kruppel-like factor 4 (KLF-4) and glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) respectively [79-81]. These targets are associated with airway smooth muscle hypertrophy and bronchial smooth muscle cells. Blue arrows reflect 3'UTR mRNA targeting while green arrows reflect indirect or unproven 3'UTR targeting.

#### **Figure 4**

Many therapeutic options for inhibition of miRNAs upregulated in disease are illustrated in Figure 4a. Anti-miRNA antisense oligonucleotides (1) can be synthesised to be complimentary to a mature miRNA of interest and inhibit its function. Modifications of these nucleic acids have been developed such as 2'-O-Methyl, 2'-O-Methoxyethyl and Locked nucleic acids (LNAs), which have additional characteristics such as increased stability in serum. miRNA sponges (2) are transcripts containing several tandem-binding sites towards a miRNA of interest, and can be stably expressed through delivery to the nucleus. A different class of oligonucleotides may be delivered to the cytoplasm to bind to miRNA responsive elements on target genes. As these oligonucleotides mask miRNA binding to gene target sites, these are aptly termed 'miRNA masks' (3).

Therapeutic options for restoration of miRNAs that are downregulated in disease are also under development (Figure 4b). Nuclear delivery (A) of DNA encoding pri-miRNA, pre-miRNA and shRNA may produce sustained restoration of miRNA expression. Transient restoration may be attained through the use of pre-miRNA and mature miRNA mimics with cytoplasmic delivery (B).

Vector	Delivery Route	Species	Disease/Area	miRNA	Inhibitor/Mimic	Reference
<b>Viral</b>						
Lentivirus	Intranasally	Mouse	Non-small cell lung cancer (NSCLC)	let-7	inhibitor	[82]
Lentivirus	Intravenous injection	Mouse	Gastric Cancer	let-7f	mimic	[83]
Lentivirus	Local Injection	Mouse	Cancer	miR-101	mimic	[84]
Adenovirus	Intranasally	Mouse	NSCLC	let-7	inhibitor	[85]
Adenovirus	Cellular inoculation	Mouse	Breast Cancer	miR-145	mimic	[86]
Recombinant adeno-associated virus (AAV)	Intravenous injection	Mouse	Hepatocellular Carcinoma	shRNA	mimic	[87]
<b>Non Viral</b>						
Naked	Intravenous and sub-cutaneous injection	Rat	Cardiac Disease	miR-208	inhibitor	[88]
Naked	Intravenous injection	Mouse	Breast/Lung Cancer	miR-10b	inhibitor	[89]
Naked	Intravenous injection	Chimpanzee	Hepatitis C virus	miR-122	inhibitor	[61**]
Naked	Intraperitoneal injection	Mouse	Hypercholesterolaemia	miR-122	inhibitor	[50]
Naked	Intravenous injection	African green monkey	Hypercholesterolaemia	miR-122	inhibitor	[50]
Plasmid	Intratumoral injection	Mouse	Colorectal Cancer	USP22 miRNA	mimic	[90]
Plasmid	Intravenous injection	Mouse	Familial Hypercholesterolaemia	Synthetic (no endogenous)	mimic	[91]

				miR)		
Plasmid- DNA-coated gold particles	Biolistic epidermal transfection	Mouse	Infection and Inflammation	miR-155	mimic	[92]
Atelocollagen-mediated	Intravenous injection	Mouse	Bone-metastatic Prostate Cancer	miR-16	mimic	[93]
Polyethylinimine (PEI)	Intravenous injection	Mouse	Colon Carcinoma	miR-145	mimic	[60]
iNOP-7	Intravenous injection	Mouse	Hypercholesterolaemia	miR-122	inhibitor	[94]
CPP targeted PEI	Intravenous injection	Mouse	Neuronal development	miR-124a	mimic	[95]

Table 1. Therapeutic strategies for miRNA modulation. Methods for therapeutic miRNA modulation *in vivo* can be broadly categorised into viral and non-viral strategies. The most popular viral delivery methods include lentiviral, adenoviral and adeno-associated virus systems. Various non-viral means of miRNA modulation exist including delivery of ‘naked’ nucleic acids or by using polymeric, liposomal or peptide targeting systems.