REVIEW

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Bottleneck limitations for microRNA-based therapeutics from bench to the bedside

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MicroRNAs are endogenous non-coding small RNAs that repress expression of a broad array of target genes. Research into the role and underlying molecular events of microRNAs in disease processes and the potential of microRNAs as drug targets has expanded rapidly. Significant advances have been made in identifying the associations of microRNAs with cancers, viral infections, immune diseases, cardiovascular diseases, wound healing, biological development and other areas of medicine. However, because of intense competition and financial risks, there is a series of stringent criteria and conditions that must be met before microRNA-based therapeutics could be pursued as new drug candidates. In this review, we specifically emphasized the obstacles for bench-based microRNA to the bedside, including common barriers in basic research, application limitations while moving to the clinic at the aspects of vector delivery, off-target effects, toxicity mediation, immunological activation and dosage determination, which should be overcome before microRNA-based therapeutics take their place in the clinic.

1. Introduction

MicroRNAs (miRNAs) are endogenous, non-coding small RNAs comprising 18-24 nucleotides, which contrast with double-stranded small interfering RNA (siRNA) produced from double stranded RNA by Dicer or direct synthesis (Love et al. 2008). MiRNAs target the expression of multiple genes simultaneously. The main mechanism by which miRNAs prevent translation of the messenger RNAs (mRNAs) transcript into a protein is generally by partial complementarity (mismatch) with the target mRNAs, which leads to decrease of the production of the protein translated by the target mRNAs (Baek et al. 2008; Selbach et al. 2008), whereas siRNAs pair to the mRNA which has a high degree of sequence complementarity with siRNA and cleavage or degrade the targeted mRNAs. Thus, miRNAs show relatively modest (~30–50% down-regulation) but widespread inhibitory effects of diverse target expression, which may correspond with their intricate involvement in the pathogenesis and therapeutic necessity of targeting multiple pathways simultaneously in diseases, rather than the high efficiency (~90% down-regulation) shown by siRNAs for their specific targets. Almost > 60% of the genes in the human genome are modulated by miRNAs (Friedman et al. 2009). Thus, miRNAs can indeed have far-reaching effects in diverse physiological and pathological processes.

In 1993, miRNAs were discovered in the laboratories of Ambros and Ruvkun. They found that a non-coding small RNA transcribed by the lin-4 gene could inversely regulate LIN-14 protein expression (Lee et al. 1993; Wightman et al. 1993). Subsequently, studies have unveiled the roles of miRNAs in specific diseases and have explored disease-specific miRNAs as potential biomarkers and therapeutic targets. MiRNA research is now a hot topic. In physiology research, many intricate mechanisms and functions of miRNAs in essential biological processes have been elucidated, such as self-renewal and differentiation of stem cells, biological developments, proliferation, replicative senescence, apoptosis, cell cycle and transportation of microvesicles (Guo et al. 2011). Significantly up-regulated or down-regulated miRNAs have been detected by several techniques, such as miRNAs microarrays, which are high-throughput tools, deep sequencing, northern blotting, and real-time quantitative polymerase chain reaction. In clinical applications, miRNA profiles, a cluster of miRNAs specific for a disease, might be detected as biomarkers of the disease for diagnosis and prognosis. With the revealing of regulatory mechanisms of miRNAs on key driver targets, pathways, and networks of cancer, viral infections, immune diseases and cardiovascular diseases, miRNAs, as a promising new category of therapeutic agents, have become interesting and worthy targets for pharmacology. Especially, anti-miRNAs, inhibitors of miRNA expression and function, have triggered enthusiasm for miRNAs as potential drug targets (Cui et al. 2013). Although in a much earlier stage than siRNA, there is hope that miRNA-based strategic therapy will be further applied in the clinic, such as cancer therapy, control of viral infection, neurological condition therapy. For instance, synthetic miRNA-145 targeted the insulin receptor substrate-1 and successfully controlled the colon cancer *in vitro* (Shi et al. 2007). Moreover, miRNA-451 mimics as tumor suppressor diminished the invasive capacity and increased cell apoptosis of human glioma cells (Nan et al. 2010).

However, are all miRNAs biologically relevant? Can each physiological and pathological process in humans be clarified by miRNA regulation? Is it true that such small entities as miRNAs can play such a large role in the body? What are the barriers to miRNAs from basic research to the clinic? Although the field of miRNA-based therapy is advancing rapidly, significant hurdles remain to be overcome. The real-world difficulties and barriers to the clinical application of miRNAs could be reflected by Roche, a large drug company, who cancelled their commitment to RNAi research (http://pubs.acs.org/cen/ news/88/i48/ 8848notw6.html). In this review, we comprehensively address the essential difficulties surrounding research into miRNAs and present new challenges for miRNAs from the laboratory to the clinic.

2. Common barriers to miRNAs in basic research

2.1. Instability of miRNAs and reproducibility of results in miRNA preparations

Currently, two methods are generally used for isolating RNA: the Trizol/TRI-Reagent isolation procedure, which is the most commonly used method for isolating total RNA, and the silica matrix or glass fiber filter-based method (Bernardo et al. 2012). During miRNA preparation and storage, the instability of miRNAs and the reproducibility of results are still the main problems that are encountered. A previous report indicated that miRNA and cDNA derivatives were highly unstable in RNA samples prepared using the TRI-Reagent method or the mirVana Isolation kit. They were gradually degraded over several days when stored at −80 °C (Bravo et al. 2007). It has also been shown that different methods of tissue collection and tissue storage for the same type of tissues results in a different quality of isolated RNA (Bernardo et al. 2012). In order to standardize miRNA preparation, Trizol/TRI-Reagent extraction, which is free of genomic DNA contamination, should be suggested as a 'gold standard' for miRNA isolation. Moreover, RNase free surroundings, including RNase free solutions, containers, tips and gloves, are essential for preventing miRNAs degradation. Finally, proper storing conditions are critical for obtaining highly stable and reliable results in miRNA isolation (Mraz et al. 2009).

2.2. Complexity of identifying miRNA targets and discerning miRNA biological functions

Target identification and functional validation is a limiting step in miRNA basic research as well as the cornerstone of miRNA-targeting therapeutic. The individual miRNA gene associated with certain disease, the variation of miRNA expression profiles of different phenotypes, and the relatively subtle change of individual target transcripts or proteins in response to the over-expression or inhibition of a particular miRNA might be detected by global miRNA microarrays, global transcriptional profiling analysis, and mass spectrometric analysis of protein expression, respectively. However, miRNA microarrays are limited by the interpretation and bioinformatic analysis of terabytes of data, and the experimental validation of downstream targets and functions remains a huge task. Computer software to predict targets by the Watson and Crick base-pairing mechanism have been developed, such as miRanda (http://www.microrna.org/), TargetScan (http://www.targetscan.org/), and PicTar (http://pictar.mdc-berlin.de/). However, the prediction is based on sequence specificity, not gene specificity (Khatri et al. 2012). Biological target prediction, assisted by bioinformatics, could predict only 50% of the regulated targets detected by a global mRNA expression microarray (Baek et al. 2008). Thus, the computer-predicted targets may incorrectly include some false-positive non-functional targets and miss some true-positive functional targets.

MiRNA regulatory networks complicate the research of discerning miRNA biological functions. Among the significantly changed miRNAs in miRNA expression profiles, which miRNAs or their combination is associated with the phenotypic change of interest, what are the targets of these miRNAs, and which signal pathways and regulatory networks are the miRNAs and targets involved in remain to be determined. In some cases, the coordinated action of multiple targets were discovered (Valastyan et al. 2009). Therefore, evaluating the biological functions of miRNAs on a specific tissue or organ is a more complex situation. The functions of each tissue or organ are determined and regulated by a series of molecules, while the expression of each molecule is regulated, not by a single miRNA, maybe by the coordinated effect of numerous miRNAs. Moreover, in addition to miRNA regulation, it is also under the control of gene mutations and modifications, methylation and other transcriptional and post-transcriptional regulations. It also has been noted that changes in miRNA expression profiles are quite different between *in vivo* and *in vitro* studies. Thus, evaluating miRNA functions *in vitro* only is inappropriate yet.

3. New challenges of miRNA-based therapy in clinical application

3.1. Bottleneck limitations: delivery and delivery vectors of miRNA modulators

The basic prerequisite of miRNA-based therapy is safe and effective delivery. There are a series of natural biological barriers to systemic miRNA mimic and/or inhibitor delivery. First, they need to traverse the circulatory system and reach the bloodstream of the target tissue, avoiding immune cell phagocytosis, endonuclease degradation, serum protein aggregate formation, and kidney filtration (kidney filtration of particles < 50 kDa). Then, they must undergo extravasation to the target tissue across the vascular endothelium (problematic for particles > 5 nm in φ; liver, spleen, certain tumors absorb particles ≤ 200 nm) and be transported into the interstitium. Finally, they must then cross the anionic cell membrane and be internalized into the target cells for final endosomal release into the cytoplasm (Bader et al. 2011; Singh et al. 2011; Wang et al. 2010). Especially, delivery across the blood-brain barrier represents an additional difficulty for miRNA-based drugs (Purow 2011).

Currently, the miRNA delivery systems mainly include chemically synthesized single stranded miRNA inhibitors, double-stranded miRNA mimics, more sophisticated systems as miRNA 'sponges'/'decoys'(containing tandem-binding sites of multiple miRNAs to inhibit several miRNAs of interest simultaneously), lipid- and nanoparticle-based delivery systems, and miRNA-expressing viral vectors (Chistiakov et al. 2012; Haraguchi et al. 2009; Qi and Mu 2012). Each delivery approach has specific limitations (Table 1).

Single stranded miRNA inhibitors and double-stranded miRNA mimics are chemically synthesized, which are cheaper and have more specificity compared with other delivery systems; however, they have poor uptake efficiency, resulting from their undesirable physicochemical properties (hydrophilic nature, high molecule weight, large size, and negative charge), which prevent them from traversing cell membranes to access target cells. Furthermore, they are unstable *in vivo* and the plasma half-lives are transient because of the presence of endogenous nucleases and immediate clearance by glomerular filtration. The

Table 1: Advantages and disadvantages of currently used microRNA delivery methods

Methods	Advantages	Disadvantages	Refs
Naked oligos direct delivery	Simple. Low-price. Less non-specificity.	Poor uptake efficiency because of undesirable physicochemical properties. Unstable. Plasma half-life is transient. Hydrodynamic injection require large dose.	Khatri et al. (2012); Wang et al. (2012); Elmen et al. (2008); Krutzfeldt et al. (2005); Song et al. (2003).
Lipid-based delivery	Stable. High efficiency of delivery.	Highly immunogenic. Brief pulse and rapidly cleared. Low efficiency of loading. Some cationic liposomes complexes inhibit the activity of serum proteins and blood cells. Some are non-biodegradable.	Khatri et al. (2012); Wang et al. (2010); De Paula et al. (2007); De Rosa et al. (2010).
Nanoparticle delivery	Stable. High efficiency of delivery. Low immunogenicity and good biocompatibility. Promising for disease target-therapy.	Complex formulation. Brief pulse and rapidly cleared.	Khatri et al. (2012); Purow (2011); De Rosa et al. (2010); Hong et al. (2011); Pereira et al. (2013); Singha et al. (2011); Boudreau et al. (2009); Chen et al. (2010); Allard et al. (2009).
Viral delivery	Long-term persistent. High-expression.	Random insertion and integration. Mutagenesis and oncogenesis. Precise cell or tissue targeting is difficult. Off-target easily. Unacceptable toxicity. Activation of immune response. Poor integration and transient expression sometimes.	Khatri et al. (2012); Purow (2011); Hacein-Bey-Abina et al. (2003); Klinghoffer et al. (2010); Ma et al. (2010); O'Loughlin et al. (2012); Yeung and Jeang (2011); Warnock et al. (2011).

stability could be partially improved by chemical modifications (Wang et al. 2012). Chemical modifications such as 2'-O methyl (antagomirs) and locked nucleic acid (LNA-antimiRs), could increase resistance of oligonucleotides to endogenous nucleases and hybridization affinity to the target sequence (Elmen et al. 2008; Krutzfeldt et al. 2005); however, the problem has only been partially resolved. Uptake efficiency and stability is also the most prominent limitation for direct delivery of oligonucleotides. Moreover, successful clinical use of antagomirs is difficult because of the large doses required (Song et al. 2003).

Lipid- and nanoparticle-based delivery systems are the two most promising vectors for miRNA delivery. The nucleic acids encapsulated in liposomes can be released into the cells effectively by fusion of the liposomes with the cell membrane; They also protect oligonucleotides from degradation by endogenous nucleases and improve the stability of oligonucleotides by coating the oligonucleotides with liposomes when systemically delivered. However, cationic liposomes were reported to be highly immunogenic. They might trigger immune response by interactions between the positively charged lipids and proteins. Intense type I and type II interferon responses and dose-dependent toxicity have been reported (De Paula et al. 2007). Nanoparticles with diameters of 50–70 nm would be a safe alternative, limiting immunogenicity, increasing biocompatibility and stabilizing complexs against degradation, such as hydrophilic polyethylene glycol (PEG) coated cationic lipid bilayers, polyethylenimine (PEI)–miRNA complexes and atelocollagen–miRNA complexes, even achieving sustained and controlled delivery to tissues, such as polylactide co-glycolide (PLGA) nanoparticles (De Rosa et al. 2010; Hong et al. 2011; Pereira et al. 2013; Singha et al. 2011). Recent reports also demonstrated that nanoparticles are promising for targeted disease therapy (Khatri et al. 2012). Nanoparticles modified with tumor-targeting scFv could deliver siRNAs and miR-34a into targeted lung cancer cells (Boudreau et al. 2009; Chen et al. 2010). Nanoparticles with ferromagnetic cores made targeted treatment by magnetic manipulation feasible (Allard et al. 2009). However, despite the great progress in nanoparticle-based delivery technologies, the brief pulse and rapid clearance observed for nanoparticle delivery, and the likelihood of the complex-induced toxicity are the limitations for miRNA applied in clinic (Purow 2011). Repeated dose delivery is needed in order to achieve therapeutic results.

MiRNA-expressing viral vectors, including lentivirus, adenovirus, and adeno-associated virus (AAV), seem attractive. These viral vectors carry miRNA expression cassettes and can provide long-term, persistent, and high expression of miRNAs after transduction into host cells. However, viruses pose a significant safety risk. Random insertion of the viral vector resulting from the integration of viral DNA into the host genome is the most worrying problem. It might disrupt normal genes or activate harmful genes, especially oncogenes and cause insertional mutagenesis and oncogenesis (Hacein-Bey-Abina et al. 2003). Precise cell or tissue targeting by miRNA viral vectors is difficult; they are prone to nonspecifically off-target and produce toxicity (Klinghoffer et al. 2010). Beside successful metastasis inhibition by intravenous delivery of anti-miR-10b cholesterol-conjugated antagomir in a mouse mammary tumor model, the alteration of liver and spleen sizes, serum proteins also was observed following the antagomir administration, which implied the potential toxicity of these miRNA modulators (Ma et al. 2010). The host immune response induced by the transduced viral vectors is another crucial problem that should be considered carefully, especially by adenovirus and AAV (O'Loughlin et al. 2012; Yeung and Jeang 2011). Moreover, occasional poor integration and transient expression (low efficiency) also hampers the application of viral delivery system (Warnock et al. 2011).

3.2. Specificity and off-target effects of miRNA regulators

One of the major obstacles in miRNA-based therapies is site specificity and off-target effects. Off-target effects, first reported

in 2003 (Jackson et al. 2003), are gene perturbations caused by RNAi molecules acting on cellular molecules other than the expected target molecules (Singh et al. 2011). The off-target mechanism has been revealed to be determined by the seed region (nucleotide position 2–7 or 2–8) nucleotide sequence located at the 5′ end of the miRNA, which is a perfect match between the antisense strand and the 3′ untranslated region of the targeted mRNA, which is essential for miRNA binding to a multitude of targets (Jackson et al. 2006; Lim et al. 2005). Off-target effects can be generated by both the sense and antisense strands, depending on their ability to be assembled onto the RNA-induced silencing complex (RISC), which is determined by their base sequence (Schwarz et al. 2003). Strictly speaking, off-target effects can be classified as specific off-target (hybridization-dependence) or non-specific off-target (hybridization independence). Specific off-target effects of miRNAs are caused by partial complementarity with non-targeted transcripts, while non-specific off-target effects depend on the chemistry or class of miRNAs, not the base sequence. In the following section, we address the specific off-targets of miRNAs and miRNA regulators.

In contrast to siRNAs, miRNAs are 'multi-targeting' and 'less-potent' (30–50%) for expression inhibition of target genes. 'Multi-targeting' means that a miRNA may target many molecules correlated with pathogenesis to help remitting or cure diseases by a particular design. For example, a single small multiple-target artificial (SMART) microRNA could be designed to target multiple members of a gene family simultaneously (De Guire et al. 2010). Conversely, it may cause unanticipated and undesired adverse effects from widespread off-target effects on hundreds of unintended cellular molecules. 'Less-potent' may mean less effect on target molecules and lower toxicity caused by off-target effects (Boudreau et al. 2009). In some cases, miRNA-mediated post-transcriptional repression is reversible (Bhattacharyya et al. 2006).

Few observations of miRNA off-target effects have been reported to date. A study confirming multi-targeting by miRNAs showed that miR-208a governed myosin switching in striated muscle cells, as well as systemic energy metabolism and high-fat diet-induced obesity (Grueter et al. 2012; van Rooij et al. 2009). Furthermore, obesity, diabetes mellitus, and cancer-related molecules were potentially linked by multi-targeting miRNAs (Ali et al. 2011). Nine miRNAs, including miR-9, miR-15a, miR-27, miR-130a, miR-143, miR-200a, miR-335, miR-375, and miR-503, are involved in the regulation of glucose and adipokine metabolism associated with obesity and diabetes, and cancer processes, including cell proliferation, angiogenesis, metastasis, apoptosis, and chemoresistance. The multi-targeting of miRNAs also poses critical issues related to off-target toxicity of miRNAs. For example, miR-29 oligonucleotide mimics, as potential anticancer drugs targeting several oncogenic pathways, have been observed to regulate immune function, bone development, and granulocytic differentiation. Systemic overexpression of miR-29 could lead to autoimmunity or myeloid hyperproliferation (Garzon et al. 2009; Li et al. 2009; Smith et al. 2012; Wang et al. 2013) (Fig. 1, C).

To date, many attempts have been made to minimize off-target effects. The recent evidence that lentivirus-mediated RNAi could reduce seed region-based off-target activity showed that lentiviral delivery might be well suited to miRNAs (Klinghoffer et al. 2010). Using a group of miRNAs targeting a single mRNA for gene silencing was suggested to reduce the dosage of the individual miRNA required for therapy, as well as the off-target activity of the single miRNA (Yeung and Jeang 2011). Moreover, miRNA reagents binding to their intended RNA targets only have been proposed, which might achieve specific and reliable therapeutic results. As well as attenuating the off-target effects of miRNA-based drugs, monitoring the global gene and protein levels, including target and non-target effects, should be considered. Nevertheless, currently, most studies of miRNAs have only focused on the intended pathological tissues and organs of interest; Toxicities and side effects in other tissues and organs have usually been overlooked, whereas safety is a vital concern when evaluating a new drug.

3.3. MiRNA-mediated drug- or chemical-induced toxicity

MiRNA-mediated toxicity has been explored recently, but only a few toxicity-related investigations for miRNAs have been reported until now. A prominent example is that the saturation of the RNAi machinery could lead to miRNA-dependent cellular toxicity. The AAv delivered short hairpin RNAs (shRNAs) competitively inhibit endogenous miRNA processing by oversaturation of the cell's RNA-transporter protein, exportin-5, in cellular sh/miRNA pathways, finally leading to liver damage and morbidity in mice (Grimm et al. 2006).

The detoxification of xenobiotics predominantly depends on metabolizing enzymes in the liver. It has been shown that miRNAs post-transcriptionally regulate the expression of drug- or xenobiotic-metabolizing enzymes such as cytochrome P450 s (CYPs) (Yokoi and Nakajima 2013). The role of miRNAs in the regulation of CYP expression was first reported by Tsuchiya et al. (2006). MiR-27b post-transcriptionally negatively regulated the luciferase activity of human CYP1B1. Subsequently, it was revealed that CYP2A3, CYP2E1, CYP3A4, and CYP24A1 are modulated by miR-126, miR-378, miR-27b, and miR-125b, respectively. In addition, CYP expression is transcriptionally regulated by nuclear receptors, which also could be modulated by miRNAs. It has been indicated that the pregnane X receptor, vitamin D receptor (VDR), human nuclear factor 4α (HNF4α), and the human aryl hydrocarbon receptor nuclear translocator are targeted by miR-148a, miR-125b and miR-27b (VDR), miR-24 and miR-34a (HNF4α), and miR-24 respectively (Yokoi and Nakajima 2013). For example, over-expression of miR-24 and miR-34a inhibited the expression of HNF4α, which is a positive transcriptional regulator of the expression of bile acid-synthesizing enzyme CYP7A1, decreasing bile acid synthesis *via* CYP7A1 (Takagi et al. 2010). Moreover, the deregulated expressions of CYPs silenced by specific miRNAs may lead to deceleration of metabolism of drugs and toxicants, followed by drug accumulation, and finally result in drug- or toxicant-induced toxicity.

A few other mechanisms of miRNA-mediated toxicity also have been revealed *in vitro*. Human miR-222 modulated matrix metalloproteinase 1 expression by targeting superoxide dismutase-2 (Liu et al. 2009). MiR-17* inhibited primary mitochondrial antioxidant enzymes (Xu et al. 2010). Let-7C is an important miRNA in cell growth and has been observed to be inhibited by a peroxisome proliferator-activated receptor alpha (PPARa) agonist, which might explain the mechanism of PPARa agonist-induced liver proliferation (Yokoi and Nakajima 2011). Circulating miRNAs may have toxicological significance as sensitive and specific biomarkers in toxicity prediction. The plasma concentrations of miR-122 and miR-192 paralleled that of alanine aminotransferase in acetaminophen-induced liver injury in mice, and the levels of miR-133a and miR-124 in plasma were associated with muscle and brain tissue injuries, respectively (Laterza et al. 2009) (Fig. 1, B).

To perfect the safety evaluation of miRNA drugs, all possibilities of miRNA-mediated toxicities in critical tissues and organs should be considered carefully. Thus, systematic preclinical pharmacology and toxicology trials *in vivo*, including general safety pharmacological assessments, special

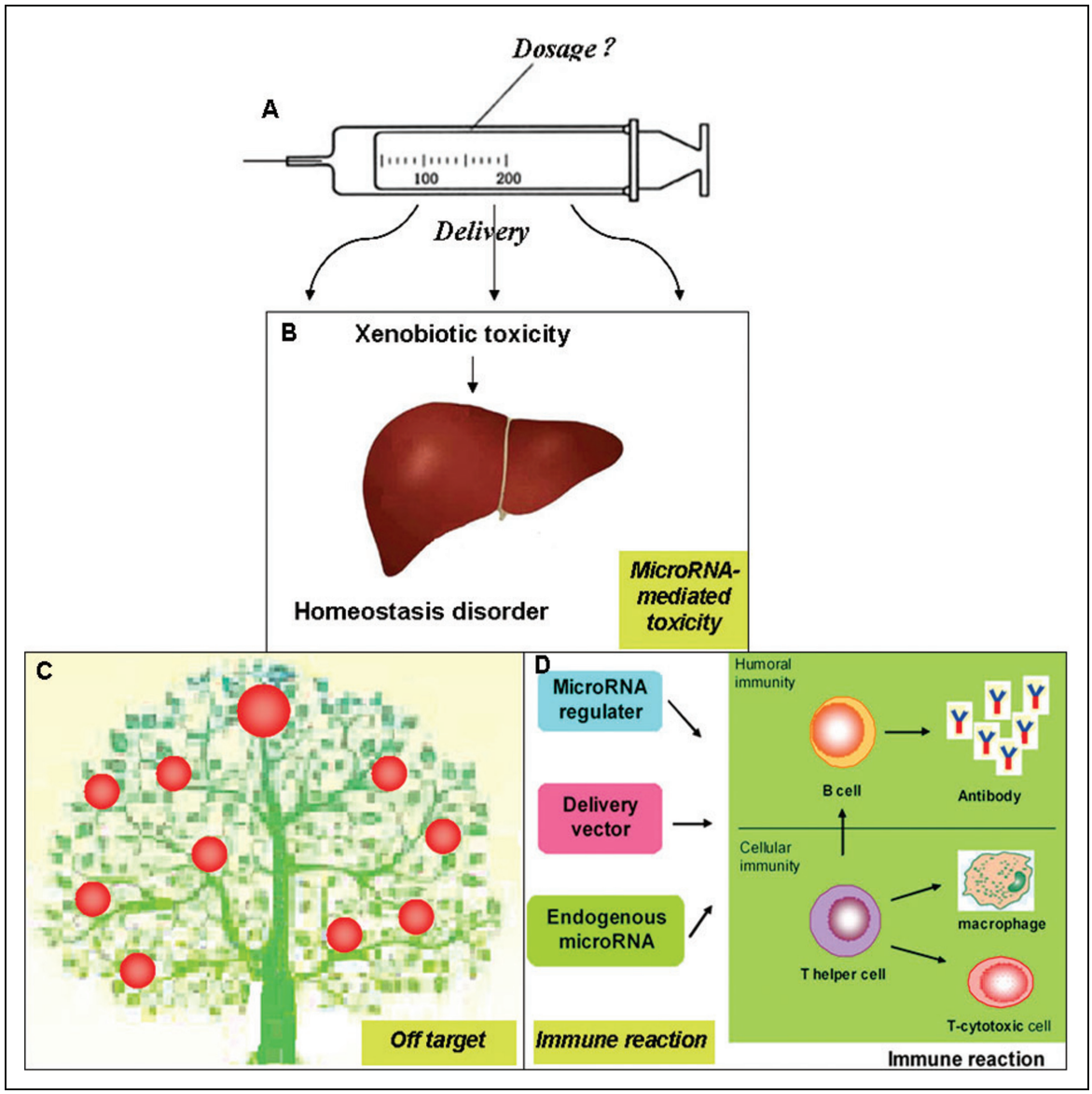

Fig. 1: The barriers to microRNA-based drugs in clinical application. There are five real-world obstacles addressed in this review, including dosage determination, delivery approach (A), microRNA-mediated toxicity (B), off-target (C), and immunological activation (D). These obstacles will be the limitative steps for microRNA-based therapy moving to the clinic.

safety pharmacological assessments (teratogenicity, carcinogenicity, mutagenicity), acute toxicities, long-term toxicities, and pharmacokinetic studies, should be carefully designed and performed. Moreover, we should be aware of the disparity of miRNA subfamilies between animal and human models when miRNA drugs firstly move to the clinic (Bader et al. 2011; van Rooij et al. 2012). Based on these concerns, special organizations, such as the Oligonucleotide Safety Working Group, have been recommended to focus on the risks and enact non-clinical regulatory guidelines (Bader et al. 2011).

3.4. Immunological activation of miRNA reagents

Immunogenicity is another major concern for the exploration of miRNA modulators (Fig. 1, D). MiRNA mimics and antagonists, as types of exogenous DNA/RNA molecules, may induce an innate immune response, which is nonspecific, and is correlated with the length, structure, chemical modification, cellular localization, and concentration, rather than the base sequence. A previous report showed that phosphorothioate oligonucleotides inhibited the intrinsic tenase complex and activated complement leading to acute toxicities (Henry et al. 2002; Sheehan and Phan 2001). Apparently, formulations may also give rise to immunological toxic reactions. For instance, liposomes may induce hypersensitive reactions, and high-efficiency delivery mediated by viral vectors into the host may generate hyperimmune responses. Recently, an RNAimmuno database at http://rnaimmuno.ibch.poznan.pl has been created to provide information on nonspecific immunological activation by RNA interference triggers and miRNA modulators. It is appreciated to reduce misinterpretation of experimental results through statistical analysis and comparisons, benefiting studies on the immunological activation of miRNA reagents (Olejniczak et al. 2012).

The immunological activities triggered by miRNA reagents might be mediated by endosomal toll-like receptors (TLRs), which could identify foreign RNA and DNA, such as bacterial and viral nucleic acids, and in turn induce interferon and proinflammatory cytokine release, leading to an inflammatory reaction and complement cascade (Barber 2011; Garzon et al. 2010). TLRs, a family of cell surface receptors, could recognize single-stranded RNA via TLRs 7 and 8, double-stranded RNA transfection via TLR 3, and unmethylated CpG motifs by TLR 9 (Judge and MacLachlan 2008; Singh et al. 2011).

Moreover, another recent study indicated that immunological off-target effects resulting from liposome-mediated transient transfection of synthetic miR-145 in human articular chondrocytes and human bone marrow-derived mesenchymal stem cells were mediated by retinoic acid inducible-gene 1 (RIG-1), not by TLRs. However, miR-145 delivered directly into the cytosol by electroporation did not cause immune gene changes, and liposomes only induced a lower immune response that was not mediated by RIG-1; therefore, it was predicted that only miR-145 transient transfection by liposomes might induce an RIG-1-mediated immune response (Karlsen and Brinchmann 2013). Another cellular sensor, NOD2, which belongs to the nucleotide oligomerization domain (NOD)-like receptors, also might recognize single-stranded RNA in certain situations (Olejniczak et al. 2012). In immunological activity assessments of RNAi and miRNA reagents by measuring the levels of cytokines, more than 70% of all papers focused on cytokines interferon (IFN)-α, tumor necrosis factor-α, IFN-β,and interleukin (IL)-6 (Olejniczak et al. 2012).

It also has been revealed that endogenous miRNAs are involved in the regulation of the innate immune reaction (Sarma et al. 2012), such as miR-511 participating in dendritic cell (DC) differentiation (Tserel et al. 2011), miR-221 and miR-155 regulating DC maturity and apoptosis (Lu et al. 2011; Ranganathan et al. 2012), miR-150 blocking early B-cell development (Zhou et al. 2007), miR-23a cluster inhibiting B lymphopoiesis, miR-17-92 cluster promoting lymphoproliferative disease and autoimmunity (Kong et al. 2010; Xiao et al. 2008), miR-181a modulating T-cell selection and sensitivity (Li et al. 2007), miR-146a controlling Treg cell-mediated regulation of Th1 responses (Lu et al. 2010). The important roles of miRNAs in immunoloregulation imply that miRNA regulators may impact the innate immune system significantly by up- or down-regulating immune-related endogenous miRNAs. Conversely, deliberate regulation of miRNAs may be applied in immunological disease therapy, especially for controlling immunological rejection in organ transplantation (Spiegel et al. 2011).

To summarize, the immune activation triggered by miRNAs would have a negative impact on the health of the recipient. A few novel strategies have been developed to reduce the likelihood of a miRNA reagent-induced immune response, such as modified nucleotides (LNAs); however, the problem has not been completely resolved. Further explorations are needed to alleviate the immunological stimulation.

3.5. Dosage concerns

Before introduction into clinical practice, dosage concerns should be addressed, because an overdose of miRNA mimics/oligos may expand specific off-target adverse effects, non-specific immune responses, and toxicities. Some recent reports demonstrated that, the overdose of intratracheal administration of synthetic CpG oligodeoxynucleotides caused acute lung injury and a systemic immune reaction (Tasaka et al. 2009); different doses of an antagomir against miR-24 led to selective silencing of miR-24 in different cells (Fiedler et al. 2011). Therefore, normal applications of miRNAs at proper physiological concentrations *in vivo* are preferred. However, animal experiments generally involved a massive overdose to achieve desirable positive results, and only the intended effects on tissues and organs of interest were observed. Thus, such an approach is not feasible and should be redesigned for clinical use. Moreover, few pharmacokinetic parameters on the processes of absorption, tissue distribution, metabolism, and excretion of miRNA modulators have been known until now, possibly because of the nature of miRNAs (Fig. 1, A).

For a miRNA drug, the saturation concentration of pharmacology, which means that a finite number of target mRNAs can be totally silenced or up-regulated by a certain concentration of the miRNA drug, should be carefully determined by titration of miRNA drugs, because over the saturation dosage, no additional pharmacology but increased toxicity and adverse reactions will produce; Moreover, the lowest efficacious dosage should be established to minimize the expansion of off-target effects and toxicities (Bader et al. 2011; van Rooij et al. 2012). Earlier work (Elmen et al. 2008; Krutzfeldt et al. 2005) showed that LNA-modified anti-miR122 oligonucleotides could exert upregulation effect of the miR-122 target aldolase A at much lower required dose of 1-25 mg/kg, compared to previously reported dose requirement of 120-240 mg/kg of cholesterol-conjugated oligonucleotides. It indicates that the proper chemical modification and advanced delivery might also decrease the lowest efficacious dosage as well as toxicity of miRNA drug (Seto 2010).

Polymorphisms in human pre-miRNAs and miRNA-binding sites have been detected. About 10% of human pre-miRNAs and less than 1% of functional seed regions of miRNAs have documented single nucleotide polymorphisms (Iwai and Naraba 2005; Saunders et al. 2007). Polymorphisms within miRNAs are highly correlated with susceptibility to diseases (Landi et al. 2008; Sethupathy and Collins 2008). The associations between germline mutations in DIS3L2, the host gene of miR-562, and Perlman syndrome, mutations in the miR-96 seed region and autosomal dominant non-syndromic deafness (DFNA50), and mutations in the miR-184 seed region and keratoconus with cataracts have been revealed (Astuti et al. 2012; Hughes et al. 2011; Solda et al. 2012). Thus, miRNA drug administration should be individualized according to the individual genotype, and the dosage regimen should be revised depending on the miRNA mutation, drug sensitivity, and metabolic rate, which are determined by genotype (Henrion-Caude et al. 2012; Wu 2011).

4. Perspectives

Despite the obstacles that must be circumvented before miRNA therapeutics can be used safely and effectively in clinical settings, miRNA technologies have been applied successfully in basic research and in experimental therapy of diseases. The most advanced miRNA therapeutic thus far is a LNA antimiR against miR-122 targeting hepatitis C (HCV) (Lanford et al. 2010). After the phenomenon that miR-122 inhibitor could reduce the HCV viral replicon RNA effectively, has been exposed, miR-122 binding sites at 5′ noncoding region of the HCV genome and the mutation studies of these sites further validated the correlation between miR-122 and HCV viral (Jopling et al. 2005). Phase II clinical trials of LNA antimiR-122 have been performed resulting in satisfactory safety and efficacy in treating HCV. This suggests that miRNAs would be promising therapeutic targets in additional disease areas; however, the study of miRNAs in pathogenesis and in clinical therapy is a young field. Nevertheless, the rapid development of miRNA therapeutics provides tremendous opportunities for gene therapy in human diseases. New breakthroughs are required to advance miRNAs from the bench to the bedside.

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