Therapeutic face of RNAi: In vivo challenges

Article *in* Expert Opinion on Biological Therapy · November 2014

DOI: 10.1517/14712598.2015.983070

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EXPERT OPINION

Therapeutic face of RNAi: *in vivo* challenges

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Introduction: RNA interference is a sequence-specific gene silencing phenomenon in which small interfering RNAs (siRNAs) can trigger gene transcriptional and post-transcriptional silencing. This phenomenon represents an emerging therapeutic approach for *in vivo* studies by efficient delivery of specific synthetic siRNAs against diseases. Therefore, simultaneous development of synthetic siRNAs along with novel delivery techniques is considered as novel and interesting therapeutic challenges.
Areas covered: This review provides a basic explanation to siRNA signaling pathways and their therapeutic challenges. Here, we provide a comprehensive explanation to failed and successful trials and their *in vivo* challenges.
Expert opinion: Specific, efficient and targeted delivery of siRNAs is the major concern for their *in vivo* administrations. Also, anatomical barriers, drug stability and availability, immunoreactivity and existence of various delivery routes, different genetic backgrounds are major clinical challenges. However, successful administration of siRNA-based drugs is expected during foreseeable features. But, their systemic applications will depend on strong targeted drug delivery strategies.

Keywords: gene therapy, small interfering RNA, RNA interference, small interfering RNA delivery

Expert Opin. Biol. Ther. [Early Online]

1. Introduction

RNA interference (RNAi) phenomenon was first reported as an unknown mechanism of silencing in 1990 [1] and finally discovered in *Caenorhabditis elegans* in 1998 [2]. It has a short but noteworthy history that represents its exceeding progress via various techniques such as knocking down genes to detect their functions (known as reverse genetics) [3-8]. Small RNAs including microRNA (miRNA) [9], piwi-like RNAs (piRNAs) and inverted repeat-associated small RNAs [3,10] all interact with RNAi pathway as a central signaling pathway [11]. Generally, RNAi is classified into two overlapping pathways based on the origin of triggering molecules; exogenous RNAi and endogenous RNAi [9,12]. RNAi strictly regulates key steps in diverse cellular processes via controlling expression of critical genes, chromatin remodeling, transgene silencing, cell:cell signaling and antiviral defense mechanism [11,13,14]. RNAi-related pathways enable cell to keep genome stability against exogenous invaders, regulate transcription and post-transcription of gene expression, and trigger cell–cell cross-talk through body fluids. Circulatory miRNAs in milk [15], blood [16], urine [17], amnion fluid and tear [18] have been repeatedly reported and are considered as novel diagnostic biomarkers in many complex diseases [19]. These circulatory small RNAs can leave cells via apoptotic bodies or exosomes to execute extracellular signal transduction [20]. After extracellular delivery of these small RNAs, recipient cells will uptake and secondary message of silencing would be induced. Additionally, cells utilize these small molecules to regulate gene expression between nucleus and organelles (e.g., mitochondria) [11].

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Article highlights.

- Diverse cellular functions including controlling expression of critical genes, chromatin remodeling, and transgene silencing are controlled via RNA interference (RNAi) pathway.
- Target-specific synthesis and efficient delivery of small interfering RNA (siRNAs) are the major challenges in RNAi therapeutics.
- Currently, leading pharmaceutical companies are challenging toxicity and safety of the technology.
- Efficient and specific delivery of siRNAs happens naturally in the body that is impressive and promising for next-generation drug development.
- Generally we can categorize challenges in three sections of biological, structural and clinical challenges.

This box summarizes key points contained in the article.

Despite of what happens in nature, scientists have been using RNAi as a quick and convenient tool in screening and monitoring gene function [21,22]. More importantly, pharmaceutical industry is looking forward to investigating diagnostic and therapeutic potentials of RNAi [23,24]. Currently, there are examples of clinical trial projects based on RNAi technology against age-related macular degeneration, herpes simplex virus-2, respiratory syncytial virus, parainfluenza virus, severe acute respiratory syndrome (SARS) and influenza type A [25], but many challenges still remain to encounter. Consequently, there should be methods to target a specific gene or family of genes within cells. Designed synthetic small interfering RNAs (siRNAs) need efficient delivery strategies. These strategies can deliver siRNAs locally or systemically to express siRNAs transiently or stably. In some cases, it is essential to target an siRNA against a special cell using specific cell surface receptors via molecules such as monoclonal antibodies.

In this review, we bring together information regarding RNAi-derived small RNA silencing pathways along with key *in vitro/in vivo* existing applications and challenges in design to delivery strategies. Then, future prospects of this technology within pharmaceutical industry are discussed.

2. Molecular and cellular mechanism

RNAi was first referred to dicing of long double-stranded RNAs (dsRNAs) into multiple small dsRNAs (**Figure 1**) via endonucleolytic activity of a protein called DICER (an endoribonuclease in the RNase III family) [26]. These small molecules known as small siRNAs can hybridize to target mRNA through a protein complex called RNA-induced silencing complex (RISC), which causes destruction or translation inhibition of mRNA [27]. The consequence is a small RNA-based, sequence-specific and homology-dependent inhibition (downregulation) of gene expression called post-transcriptional gene silencing (PTGS) [11]. Furthermore, other complexes were also identified that can migrate into nucleus to induce DNA methylation and chromatin remodeling along with expression silencing regulation termed as transcriptional gene silencing (TGS) [11]. Despite small RNA-mediated TGS, transcriptional activation is also investigated via a mechanism known as RNA activation (RNAa) [28].

In addition to gene expression regulation within cells, extracellular transfer of small RNAs in *Arabidopsis* [14] and silencing inheritance pattern in offspring of *C. elegans* is also reported [13]. This indicates strong relationship between RNAi and epigenetic regulation of gene expression. Interestingly, gene silencing and activation in human are also associated with extracellular transport through blood as they have been reported in more than 10 body fluids [11,29]. This phenomenon can be cited as a novel mechanism of gene expression regulation and extracellular signal transduction.

Basically, RNAi is a core system for gene silencing pathways (**Figure 2**). Different small molecules have the ability to activate this conserved pathway and are generally called noncoding small RNAs. The well-known examples in human are miRNAs, siRNAs and piRNAs. These small molecules have different origins and can induce the pathway through different branches. Cytoplasmic dsRNA molecules are considered unusual and are substrate for endonuclease DICER, HIV-1 TAR RNA-binding protein (TRBP) and protein kinase R-activating protein (a protein activator of the interferon-induced protein kinase: PACT) help Dicer to identify and dice dsRNA into about 21 bp fragments with 2 nucleotides (nt) overhangs at each end (**Figure 1**) [30]. This overhanging structure works as a signal for RISC to detect and bind to these small RNAs. Argonaute (AGO), an important enzyme within RISC complex, plays a critical role in processing procedure [31]. Once RISC binds to small dsRNA (siRNAs, miRNAs or piRNAs), it chooses one strand of loaded small RNA as template to screen mRNAs. During screening process, a full or partial complementary match will induce complete target cleavage or ribosome inhibition, respectively. Thus, target gene expression will be interrupted. PACT and TRBP also company with AGO [11]. In some cases of siRNA-induced translational suppression, RISC sequesters target mRNA sequence and migrate toward special places called processing bodies (p-bodies) [32]. This action happens when cells need to reread arrested mRNA molecules in some other conditions. If so, these molecules are transferred back into cytoplasm for translation and RISC unbounds from target mRNA (**Figure 2**).

Among all kinds of small noncoding RNAs, miRNAs are in greater importance. miRNAs are transcribed from genes located inter- or intragenic regions via RNA polymerases. After transcription, miRNA transcripts are called primary miRNAs (pri-miRNAs) with hairpin structures. Within the nucleus, Drosha (RNase III family nuclease), DiGeorge critical region 8 (a double cysteine-ligated Fe (III) heme protein: DGCR8) and histone deacetylase proteins (called microprocessor complex) recognize and cleave pri-miRNA with a special chemico-structural pattern [33,34]. The processed

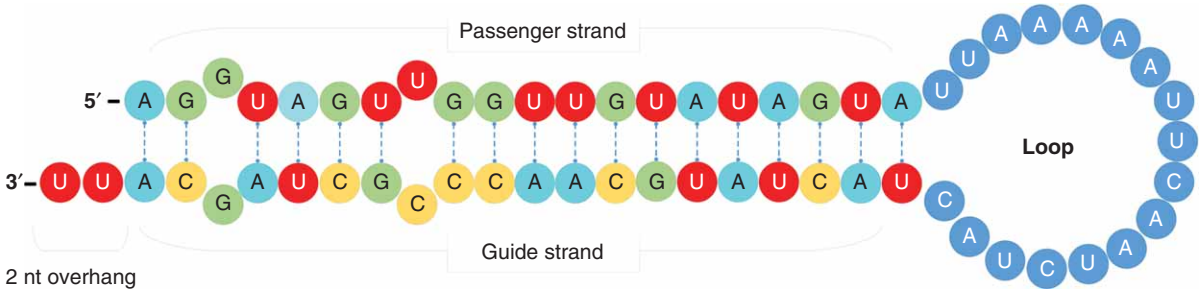

Figure 1. Simplified structure of a microRNA/siRNA illustrating structural features.

pri-miRNA is called precursor miRNA (pre-miRNA), which has 2 nt overhang in 3′ end. This pattern is also recognizable with Exportin-5 and Guanosine-5'-triphosphate (GTP) binding nuclear protein Ran (RAs-related nuclear protein: RanGTP) proteins that transfer pre-miRNA into cytoplasm [35]. DICER can recognize pre-miRNA molecule in the cytoplasm. In addition to mentioned paths, some miRNAs follow other routes. For example, some miRNAs are intronic (called mirtrons), which are released after splicing [36], some are derived from special tRNAs [37], and some doesn't need preprocessing and can be directly loaded to RISC complex [38]. Also, some viruses encode miRNA genes, which enables misregulation of host gene expression systems via RNAi pathway [39].

Finally, there are other germline-specific small RNAs called piRNAs, which suppress transposable elements and maintain genome integrity [40]. Their generation and pathways are not well known but are critical in suppression of transposable elements. In addition to miRNA, siRNAs are other small noncoding RNAs with extracellular origin and can be generated from two paths. The first class of siRNAs is generated from direct DICER activity on viral dsRNAs during viral replication process. The second category is related to delivery of these molecules through genetic engineering and drug delivery techniques, including direct dsRNA or siRNA delivery into cells as transient or stable silencing strategies. These observations suggest that there is tremendous potential in drug delivery and controlled expression of target genes for the foreseeable future. In the following sections, siRNA synthesis and common delivery strategies will be uncovered in more details.

3. SiRNA structure, design and synthesis

SiRNAs are short 21 – 23 bp dsRNA including 2 – 3 nt 3′ overhangs and 5′-phosphate groups [21]. The 5′-phosphate groups play a critical role in antisense strand recognition and incorporation of RISC [41]. Since there are diverse kinds of small noncoding RNAs, they are also diverse in stability, functionality and structural features. For instance, circulatory miRNAs are very stable against RNases. They also transduce regulatory signals between cells as an alternative to cytokine/chemokine and hormone/receptor mechanisms. Therefore, designing siRNAs can be a simple sketch or complicated chemical modifications. More findings about natural mechanism of silencing including siRNA cellular uptake and RISC activation will lead into more optimized design of siRNAs for more efficient silencing process.

Different biochemical studies have been developed in order to improve stability and functionality of synthetic siRNAs. Special chemical modifications are reported to enhance stability and efficacy, reduce off-target effects, and avoid immunostimulatory effects of siRNAs [42]. Therefore, several steps must be considered in siRNA design and delivery strategies, including stability, specificity, facilitated loading of siRNAs into DICER/RISC [43]. Also, siRNAs may have endogenous or exogenous origins, which highly depend on efficient targeted delivery methods. Endogenous siRNAs provide stable silencing but exogenous ones can trigger either transient or stable functions. In most cases, transient silencing is carried out with direct application of synthetic siRNAs [44] which lasts up to 2 – 3 days depending on cell line, proliferation rate, transcription level of targeted gene, siRNA dose and delivery efficacy [45].

However, RNAi is a potent technique for *in vitro* studies, but there are some challenges for *in vivo* applications. Applications including gene function analysis, target identification and validation, and therapeutic agents are the main spots of this new technology. Interestingly, RNAs have many two-dimensional (2D) and 3D structural differences with DNA. They can make non-Watson–Crick complementary interactions such as guanine:uracil (G:U) and adenine:cytosine (A:C) that are called mismatches [46,47]. Therefore, siRNAs have specific characteristics in binding to their targets and our current limited knowledge about siRNA incorporation to RISC and target finding mechanism cause mistakes and therefore off-target effects, including nonspecific silencing of unwanted genes and dose-dependent immunogenic response [48]. Additionally, it is extremely complicated to avoid the off-target effects due to spatiotemporal gene expression pattern of these molecules [49]. Furthermore, age, sex, tissue, organ, tumor and individual specific specificity should be also considered as other variables [50]. As a conclusion, prediction of susceptible off-target domains that can influence silencing efficiency is the first step [46,49]. Some studies

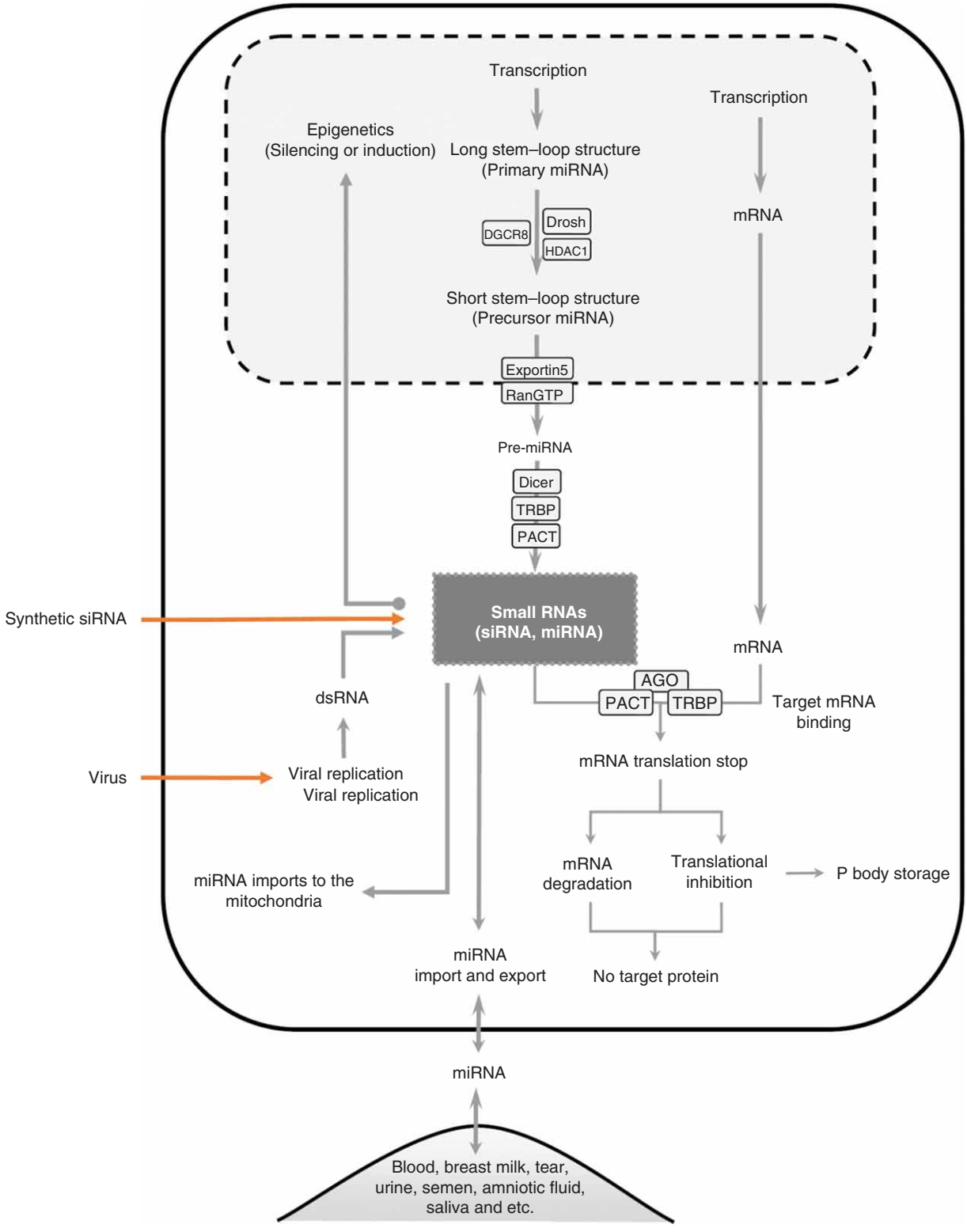

Figure 2. Cellular and molecular pathway for biogenesis of microRNAs and its interconnection with siRNA pathway. Endogenous miRNAs are transcribed into a long hairpin structures known as pri-miRNAs. Then, Drosha cleaves these huge molecules to smaller hairpins called pre-miRNAs. Mature miRNAs are generated by catalytic activity of Dicer on pre-miRNAs within cytoplasm. Cytoplasm is a pool of small noncoding RNAs including miRNAs, siRNAs and piwi-like RNAs, which some of them can pass mitochondria and plasma membrane. Viral genomes are also coded for small RNAs and can regulate gene expression of hosts. This small RNA pool can bind to target mRNAs within cytoplasm. This hybrid triggers an enzymatic complex known as RISC that degrades the target or blocks translation. Some of blocked hybrids are stored in special cytoplasmic areas termed P-bodies. During specific conditions these stored small RNAs are delivered back to cytoplasm.

AGO: Argonaute; HDAC: Histone deacetylase; PACT: Protein activator of the interferon-induced protein kinase; TRBP: TAR RNA-binding protein.

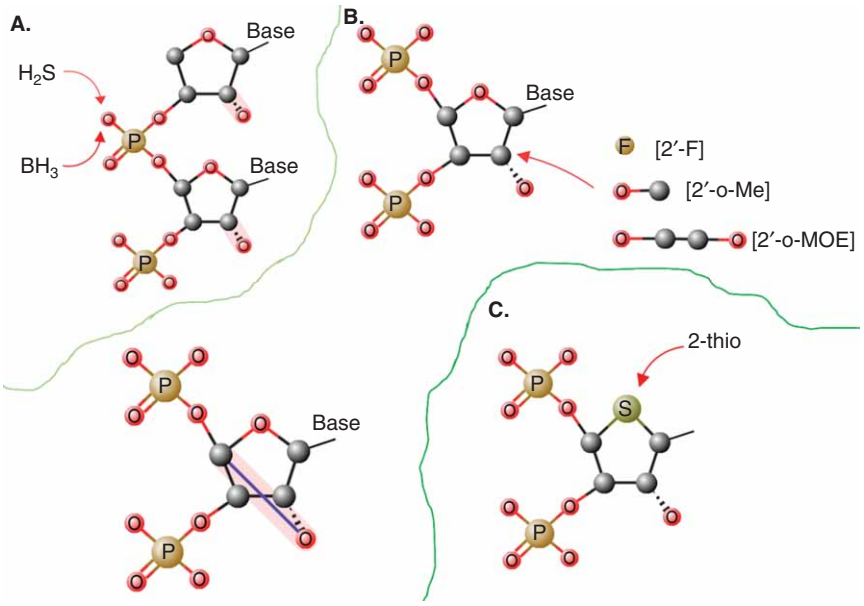

Figure 3. Different substitutions and derivatizations in (A) phosphate bridges, (B) sugars and (C) bases of nucleosides.

recommend utilization of more sensitive alignment algorithms or siDirect instead of BLAST database [47,48,51] to predict a target:siRNA matching without cross-reactivity [52]. Beside their rapid, potent, specific inhibitory functions, targeting gene families using a single siRNA is another interesting characteristic of siRNAs-based drugs [53-56].

Generally, there are three most popular chemical modifications on siRNA structure including modification of the phosphodiester backbone, ribose 2'-hydroxyl group (R-2'-OH) and ribose ring. Host endonucleases can easily digest phosphodiester bond in RNA backbone (**Figure 3A**) [57]. In order to increase stability, oxygen bridges of RNA backbone can be replaced with *phosphorothioate,* alternatively. However, it would increase toxicity and reduce silencing activity [58-62]. Another alternative is *boranophosphate linkages* that are more nuclease-resistant and less toxic compared with phosphorothioate [63]. Also, *phosphonoacetate linkages* are completely resistant to nuclease degradation and are electrochemically neutral (if esterified) [64], which facilitates absorption of modified oligonucleotides by cells even in the absence of delivery reagents [65].

Besides phosphodiester bonds, 2'-OH group of ribose initiates cleavage-transesterification process through a nucleophilic attack on phosphodiester bond under specific conditions, which is followed by hydrolysis (**Figure 3B**) [44,66]. Frequent modifications have been carried out on 2'-OH to resolve this problem through substitution or blocking of this functional group. The most widely used substitutions are 2'-*O*-methyl (2'-OMe) and 2'-fluoro (2'-F), which both increase nuclease resistance and thermostability of the structure [62,67], while may reduce potency of molecules in some cases [41]. 2'-*O*-methoxyethyl (2'-O-MOE), 2'-*O*-alkyl and other bulky groups may improve antinuclease shield of siRNA but such substitutions are less tolerated on positions like 3' overhangs. However, disturbing thermodynamic asymmetry of siRNA by addition of 2'-aminoethyl at 3' end of passenger strand improves efficiency [68,69]. Also, lower thermodynamic stability in 5' end of antisense (guide) strand than sense, which binds to mRNA nonregulatory regions, avoids interference with regulatory proteins [70]. On the other hand, 2'-OMe modifications of U and G nucleotides can reduce immunogenicity of synthetic siRNA [71]. Alterations in sugar compartment of nucleotides result in reduced flexibility and nuclease sensitivity of siRNA structure. Binding of ribose 2'O into 1'C (producing Oxetane) [69,72] with methylene bridges results in a locked conformation called *bridged nucleic acid.* Such alterations in sugars bring about new generations of nucleotides called *locked nucleic acid* (LNA) [73] because of inability to project all kinds of possible configurations. *In vivo* nuclease resistance of this structure is enhanced [74,75] but just 4 – 6 simultaneous modifications are tolerated in siRNA structure [76]. In contrast, utilization of derivatives of RNA without C2'–C3' sugar bonds, called *unlocked nucleic acid,* destabilize widely modified siRNA in contrast with LNA [77,78]. On the other hand, substitution of pentose with hexose monosaccharaides such as cyclohexenyl, anitrol and arabinose, respectively, was applied to develop *CeNA, ANA and 2'-F-ANA* (products of cyclohexenyl, anitrol and arabinose, respectively) [79,80] that result in enhanced stability of siRNA *in vivo* [81].

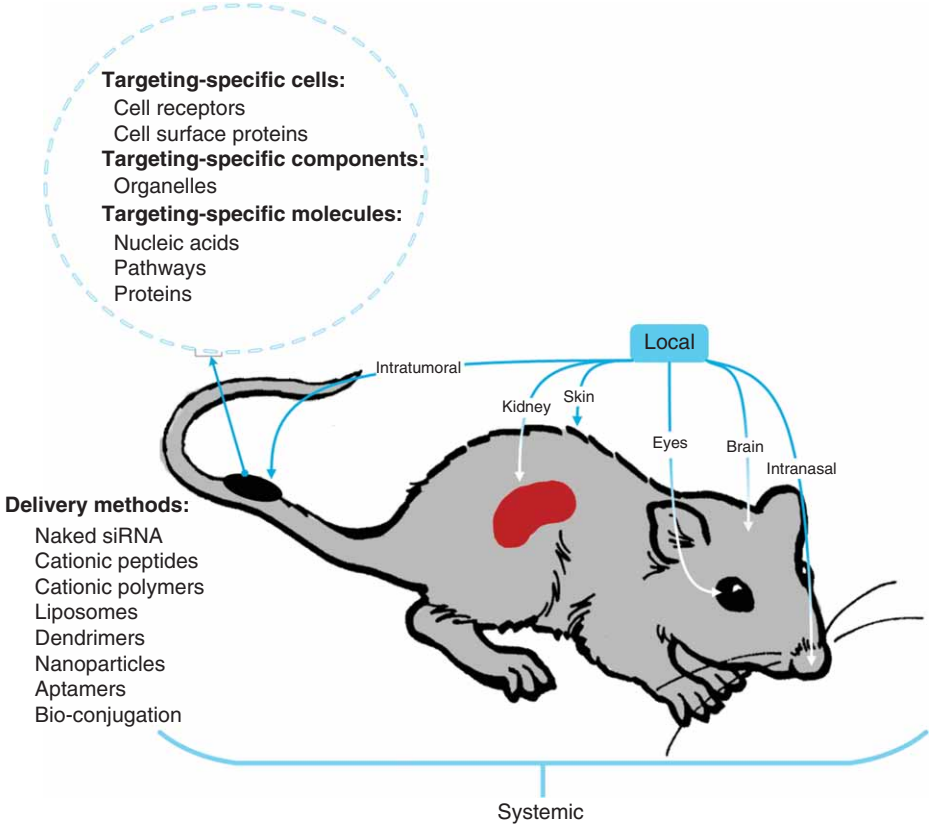

Figure 4. Schematic view of *in vivo* siRNA delivery strategies including local or systemic approaches.

Manipulation of nucleotide bases in order to increase stability and protein interactions can be the last step for structural improvement of siRNAs. Modifying bases with *thio, hydroxy and iodo* in specific places of nucleotides or utilization of pseudo uracil bases in siRNA structure would increase potency of siRNA and among, just *2-thio* and pseudo uracil have presented more efficient functions (**Figure 3C**) [82].

4. Delivery strategies

Favorable pharmacokinetic properties, nontoxicity, specific effects of carriers or their components, and absence of cross-contaminations are main concerns in drug delivery strategies [83]. Additionally, drug payload protection, organ/cell-specific delivery, proper cellular uptake and finally correct intracellular localization are of other concerns [84]. Therefore, several *in vitro* and *in vivo* drug delivery methods have been developing [85].

Targeted delivery of siRNAs appears to be key step in accelerating RNAi-based high-performance treatments. Extracellular induction of RNAi pathways encounters several obstacles and their targeted delivery appears to be a critical step. Initially, the molecule must have positive net charge within normal physiological conditions in order to interact with and diffuse through cell membrane [86]. Generally, mediators or carriers such as liposomes and dendrimers are supposed to prepare a cationic coat around synthetic siRNAs to reduce nuclease activity and improve cell delivery [84].

The simplest delivery strategy is systemic administration of naked and free of transfection reagents siRNAs, where cells are directly treated with the least toxic siRNA concentrations [87]. Also, other techniques such as microinjection and electroporation have also been developed for direct delivery of siRNA but exhibit high levels of toxicity [88].

Also, various administration routes (including intravascular, intratumoral, intracranial, intraperitoneal, intrasplenic, intramuscular, subretinal, subcutaneous, mucosal, topical application, and oral ingestion) have been considered in order to develop or improve delivery strategies (**Figure 4**) [89]. However, each transfection process needs to be optimized for cell density, siRNA concentration, transfection reagents and so on [90]. **Figure 4** shows a classification of different delivery strategies that are discussed in following sections.

4.1 Systemic or local siRNA delivery

As the first step, synthetic siRNAs must be modified and formulated to adopt efficient delivery rates. There are two areas of concern, including siRNA (sequence, features and structure) and its delivery strategies (local or systemic). Delivery strategies vary from traditional systemic utilization of naked

dsRNA to modern nanoencapsulation, complexations with polycationic polymers and peptides, and derivatives of sterols or conjugation with cell surface receptors [91]. Systemic delivery imposes several requirements and greater hurdles than local *in vivo* delivery since all cells will be exposed. Nowadays *in vivo* studies are applying systemic administration strategies to evaluate global outcome of siRNA therapy [92].

Applying systemic delivery methods need high-quantity of siRNAs [93]. In contrast, localized delivery requires less concentrations and subsequently do not impact whole organism [89]. Recently, various delivery strategies are developed including liposome and polymer-based nanoparticle encapsulations or antibody, peptide, aptamer, and cholesterol (Chol) conjugations to improve targeted and systemic delivery of siRNAs [94]. Both systemic and local delivery strategies can be further accomplished via targeting against a special cell component such as surface receptors [95].

4.2 Delivery techniques

Generally, siRNA-based researches use two methods to attain silencing responses, including employment of special plasmid and viral vectors (generating siRNAs using host's expression system) or direct delivery of synthetic siRNAs (direct incorporation of RISC).

4.2.1 *Viral carriers*

siRNAs can be efficiently delivered by hijacking viruses such as *lentiviruses* and *adenoviruses*. However, industrial-scale production along with safety risks such as conceivable mutagenic and immunogenic issues are still critical challenges [96]. In some cases like genetic diseases or disorders where stable silencing is preferred [97], plasmid or viral vectors are applied that can generate mature siRNAs for RISC or small hairpin RNA (shRNAs) for DICER. Transfection of these vectors is mainly performed by electroporation [90]. A mature platform in development of different versions of silencing is viroid that target specific cell lines. Adeno-associated viruses, adenoviruses (e.g., Ad5), retroviruses (e.g., MoMLV and lentiviruses) and herpes simplex virus are among widely used viral vectors [98,99]. Lentiviral and adenoviral let7 antagomiRs (also known as blockmirs or anti-miRs) has been used to target NSCLC through intranasal delivery methods in murine [73,100]. However, viral bodies stimulate immune system, which leads to development of virosomes. Low increase in antibody level and specificity along with nontoxicity can be mentioned as advantages of virosomes in comparison with other vectors [101].

Consequently, low immunogenicity, no internal recombination, high reproducibility are critical advantages of non-viral siRNAs in comparison with viral carriers [102]. Additionally, higher packaging capacity, extensive and more flexible modifications, biodegradability, higher blood solubility, lower toxicity and ease of synthesis are other advantages of non-viral carriers [103].

4.2.2 *Non-viral carriers*

Naked oligonucleotides and siRNAs could easily be degraded within body. Therefore, therapeutic activation/inhibition of expression could not be achieved via direct administration of siRNAs *in vivo*. Hence, different delivery vehicles have been designed to overcome natural barriers [104]. Often stable or transient silencing depends on specific carrier to be accomplished. Besides, biocompatibility of the carrier and period of silencing also must be considered.

SiRNA delivery strategies consist of four main categories including: i) naked; ii) lipid-based; iii) peptide-based; and iv) polymer-based delivery methods [103]. Naked engineered siRNAs can be administered directly (systemic or local) without any possible protections especially for cell culture tests. Lipid-based methods consist of conjugation of lipids with antibody, peptide and particles that engulf siRNA. Basically, polymer-based methods are similar to lipid-based methods in targeting, except some special triggers such as temperature, pH or pulse release can be improvised inside scaffolds [105].

4.2.2.1 *Cationic peptides*

Peptides are small fragments of proteins that may have secondary or even tertiary structures with specific features and charges. Positively charged peptides can interact with membranes. Cell penetrating peptides (CPPs) are those cationic peptides that can penetrate into cells. CPPs can interact covalently or noncovalently through disulfide or electrostatic/hydrogen interactions with siRNAs, respectively [106]. HIV-1 trans-activator of transcription was the first peptide discovered to penetrate cell membranes [107]. Later, viral protein (VP22) [108], MPG (a peptide vector) [109], model amphipathic peptide [110] and poly-arginine [111] were discovered with the same abilities. Small cationic polypeptides (poly His, Lys and Arg) coat and neutralize siRNA to pass through membrane [112]. Although, naked siRNA delivery strategies have special advantages but cationic carriers with high solubility and low toxicity show better results in targeted siRNA delivery [113]. Knockdown of superoxide dismutase, caspase 3 (Casp3) and Casp9 in primary neuron cells through uptake of penetratin–siRNA conjugate [114], green florescent protein (GFP) transgenic injected mice with GFP siRNA/Rabies virus glycoprotein-R9 (RVG-R9) or siRNA/Rabies virus-matrix protein-R9 (RV-MAT-R9) [115] demonstrated significant, low toxic and targeted knockdown of target genes.

4.2.2.2 *Cationic polymers*

Cationic polymers can envelope and incorporate with negatively charged molecules to neutralize membrane-molecule disfavored interactions. Cells phagocyte membrane interacting carriers and disintegrate them inside endolysosomes [116]. Chitosan, gelatin, cationic dextran, cationic cellulose, and cationic cyclodextrin and some synthetic biocompatible cationic polymers including polyethyleneimine (PEI), poly-L-lysine (PLL), poly(amidoamine)s (PAAs), poly(amino-co-ester) and

poly(2-*N*,*N*-dimethylaminoethylmethacrylate) are widely used natural biodegradable, biocompatible and less immunogenic cationic polymers [117]. PEIs with low molecular weight are suitable for destabilization of lysosome membrane. On the other hand, poly L-lysine, which is one of the first polycationic polymers for preparation of polyplexes, has low buffering and transfection capacity and can undesirably interact with blood proteins that make it more toxic as increased size [103,118]. Chitosan, gelatin, dextran, poly(D,L-lactide-co-glycolide), and polylactide nanoparticles are biodegradable polymeric carriers that release encapsulated siRNA during gradual degradation or digestion [112]. In some cases, neutral polymers such as PEG can optimize and enhance solubility of siRNA in systemic delivery. Lactosylated PEG–siRNA (Lac-PEG–siRNA) conjugate is recently synthesized and successfully utilized *in vivo* [119]. Also, hypoxia-inducible factor-1α conjugated with transferrin–PEI (Tf–PEI) complex is used to deliver shRNA-vectors to mice cells and reduced tumor growth [120]. Reducible poly-cationic polymers are another member of this family that release their contents during various adopted conditions including pH or temperature fluctuations or according to buried in reducible residues [121,122]. For example, insertion of acid-labile b-thiopropionate bond between siRNA and PEG facilitates acid-triggered intracellular release of siRNA within the cytoplasm [123].

It has been reported that tail-to-tail linkage of small amphiphilic oligopeptides can make special open-ended nanotubes with self-assembly properties. These peptides are beneficial for siRNA encapsulation and delivery [124].

4.2.2.3 Liposomes

Liposomes are double layer vesicles made of generally bipolar lipids. Since amphipathic phospholipids can encapsulate both hydrophilic and lipophilic compounds based on their bipolar structure, these carriers have broad applications for different drug natures. Positioning of drugs in liposomes depends on dispersion ratio (preference of any molecule to interact with hydrophobic or hydrophilic molecules) of drug in aquatic and lipidic phases (**Figure 5**). Moreover, negative net charge can be induced on liposome membrane using phosphatidylserine, phosphatidylinositol, phosphatidylglycerin and stearylamine molecules within liposome membrane [125]. Generally, liposomes are classified into three classes, including MLV, small unilamellar vesicles and large unilamellar vesicles with respective 0.5 – 20 μm, 25 – 100 nm and 100 – 500 nm diameters [125]. Also, liposomes are developed for passive or active targeting mechanisms in different complexes of liposome and other interacting molecules including lipoplex, liposome polycationic DNA, Man-liposome and so on. [126]. Among all, cationic liposomes have been more efficient in delivery and transfection.

In contrast to usual liposome administrations, plasmid DNA (pDNA) such as antigen-encoding plasmid DNA has been used in cancer gene therapy [127]. Administrating cationic liposome–pDNA complex (lipoplex) raises immediate immunogenic aggregation reaction [128]. Mahato *et al.* [129] demonstrated that cationic liposome [32P] pDNA complexes are rapidly cleared from blood circulatory system and accumulated extensively in lung and liver [128]. However, inhibition of liver non-parenchymal cells (NPC) uptake of [32P] lipoplexes following administration of dextran sulfate, suggests involvement of a phagocytic process [130,131]. Intravenous and intranasal effects of both naked and complex with cationic liposomes siRNA are compared in C57BL/6 mice, which revealed high impact of cationic residues [132].

Furthermore, functionalization of liposomes with some surface manipulations may increase targeted transfection efficacy. Also, transfer of siRNA with mannose (Man)-coated liposomes would be useful for treatment of some cancers especially liver and brain cancers. Man-C4-Chol has positive net charge that is induced on surface of a Man-liposome and enhances electrical interaction with gene or siRNA [133]. Since siRNA expression with Man-lipoplex can be significantly reduced in liver by predosing of mannosylated bovine albumin serum, a mannosylated carrier is preferred for NPC-selective siRNA transfection [134]. Basically mannose-coated liposomes and galactose (Gal) liposomes (Gal-liposome) are effective and efficient for siRNA transfer. Radioactive emission from Gal-liposome [32P] pDNA complex (Gal-lipoplex) is about 75% of applied dose even 1 min after intraportal administration in liver cancer cells. Downregulation of hepatic gene in Gal-lipoplex-treated cells is 10-fold greater than bare cationic liposomes [135]. Currently, liposomes are widely used for *in vitro* and *in vivo* experiments successfully due to resemblance to basic components of cells. Accordingly, different products such as Lipofectamine (Invitrogen) and OPTIMEM (GIBCO) have been commercialized and routinely being used [136].

4.2.2.4 Dendrimers

Dendrimers are hyper-branched, tree-shaped and 3D structures. The branches (dendritic structures) are attached to a central core. Also, functional groups are bound to its exterior surface (**Figure 5**). Utilizing broad spectrum of functional groups makes it possible to synthesize dendrimers with extensive applications. These characteristics along with controlled weight, composition, pharmacokinetics and biocompatibility of dendrimers make them suitable for systemic delivery of siRNA [137]. There are different classes of cationic and anionic dendrimers such as poly(amidoamine) (PAMAM), poly(propylene imine), PEG-grafted carbosilane [138]. In comparison with cationic liposomes, dendrimers endure better *in vivo* half-life [139] and are smaller in size [140] while lipid complexes are rapidly cleansed from blood circulation due to their huge size [141]. Specific dendritic polymers like PAMAM have been widely utilized in *in vivo* drug delivery [142,143]. Conjugation of Tat peptide (GRKKRRQRRRPQ) with PAMAM-G5 can efficiently inhibit multi-drug resistance-1 gene expression *in vitro* [140]. PEGylated PLL conjugated dendrimers were successfully utilized against human HT-29 and murine

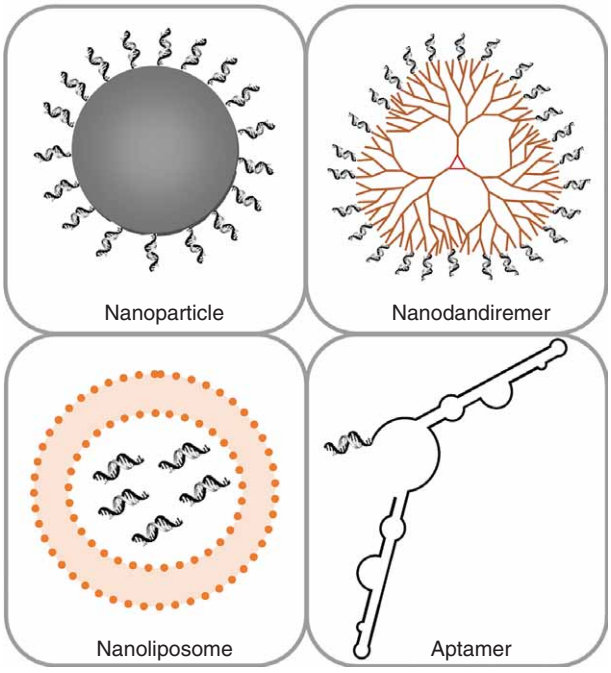

Figure 5. Schemes of different carriers and structures. Nanoparticles, nanodendrimers, nanoliposomes and aptamers are simple examples of siRNA carriers.

C26 colon carcinoma models with acceptable survival rate [144]. Also, capping PLL dendrimers with methotrexate enhances stability and decreases toxicity [145]. Kaminskas *et al.* studied attachment of doxorubicin with a pH labile linkage to release the drug from PEGylated dendrimers in comparison with PEGylated liposome, which demonstrated low toxicity, better distribution and release of the drug [146].

4.2.2.5 *Nanoparticles*

Nanomaterials with < 100 nm in diameter resemble (e.g., their size, structure) natural cell components such as surface or soluble receptors. Nanoparticles can easily interact with molecules. In some cases of systemic delivery it is needed to use a targeted nanocarrier–siRNA complex. Pack *et al.* have accomplished an experiment to condense DNA or RNA into cancer-targeted nanoparticles with PEI, PLL and cyclodextrin-containing polymers (**Figure 5**) [147]. Furthermore, Gao *et al.* demonstrated better stability and distribution of modified siRNA nanoparticles *in vivo* [148]. Also, PEI–PEG–Arginine-Glycine-Aspartic acid (RGD) fusion architecture was used to inhibit VEGFR-2 expression [149]. Angiogenesis can be inhibited by downregulation or silencing of VEGFR-2 expression [150]. Intravenous injection of siRNA-nanoparticles into BALB/c mice with N2A tumors (located at four to six different regional lymph nodes) presented tumor uptake, proceeding inhibition of protein synthesis, angiogenesis and tumor growth [149].

Micelle is another suitable nanostructure for siRNA delivery that can be conjugated with stabilizing materials such as PLL, PEG or poly-alkyl cyanoacrylate [151]. The spherical nanoparticles (with an average diameter of 117 nm) of polyelectrolyte complex (PEC) micelles [152] are prepared based on interaction of PEG and siRNA conjugated with PLL (PLL–PEG) [153]. PEC micelles show high RNAi activity against HuH-7 cells [154]. DNA nanocarriers, which are classically categorized as nanostructures, are also utilized in various forms like Box, WF Prism, WF Tetrahedron, WF Buckyball, or Tubes to deliver different types of molecules such as siRNA [155]. On the other hand, PEGylation of nanoparticles cause a phenomenon called 'muco-inert' properties, which enhances diffusion process through mucus and peptidoglycan barriers [156,157]. Any kind of polymers and macromolecules with < 100 nm dimensions are classified as nanoparticle, which provide enough equipment to properly transfect different cells.

4.2.2.6 *Aptamer*

Aptamers are tertiary structured DNA and RNA molecules that can interact with specific proteins or small molecules like antibodies. In comparison with proteins, aptamers have higher degree of freedom in bonds. These structures can be folded to recognize objects in higher resolution [158]. SiRNAs can be coupled with aptamers or oligodeoxynucleotide through a disulfide bond. This enables release of active into targeted cells siRNAs proceeding cytosolic uptake (**Figure 5**). Conjugate of aptamer–siRNA may represent a novel class of therapeutics with widespread applications in medicine [159].

4.2.2.7 *Bio-conjugation*

Bio-conjugation is not departed from usual previously mentioned carriers but a careful combination. Adaptation of different coating delivery systems with site-specific molecules such as antibodies, provide a raw platform to introduce new virus-like delivery systems. Here, some classical successful *in vivo* examples are presented. RGD peptide ligand, VEGFR (especially VEGFR-2) and mannose receptors are good examples of cell surface receptors to be targeted with combinatorial systems [160]. For example, RGD containing peptide sequences can interact with integrins that are upregulated in neovasculature [161]. Any delivery system can be conjugated with these markers to increase the accuracy and efficacy of drug accumulation in targeted areas. RNA and Chol conjugate has been experienced for expression inhibition of apolipoporotein B in mice [162]. Also, anti-human epidermal growth factor receptor-2 antibody conjugated with PEI, decrease growth rate of human breast cancer cells [163]. Also, an ovarian cancer delivery system including PEI, PEG and Fab fragment of monoclonal antibody against integrin-associated protein, known as OA3, was utilized to increase rate of transfection in cancer cells [152]. In another study anti-Anti-C1q antibody (JL-1 antibody) conjugated with PLL enhanced transfection efficacy of leukemia cells. Using this strategy, cells could be

Table 1. Some non-viral carriers of engineered siRNA into lungs *in vivo* indicating successful *in vivo* studies which show different genes and delivery applications.

Carrier	Target	Model	Effect	Ref.
Cholesterol peptides	MAP Kinase	BALB/c mice	P38 MAP kinase knockdown in target tissue	[184]
Lipid based	siGLO Green	B57BL/6 mice	Detection of siRNA in target tissues	[181]
Cationic liposome	Cadherins	B57BL/6 mice	Detection of siRNA in target tissues	[185]
PEI	eGFP	1Osb/j mice	Decrease in GFP expression in target tissue	[186]
Cationic liposome	siGLO Red	Athymic nu/nu mice	Longer detection of siRNA in target tissue	[187]
PEI	eGFP siGL3	B57BL/6 mice	Successful knockdown of genes in target tissue	[188]

GFP: Green florescent protein; MAP: Model amphipathic peptide; PEI: Polyethyleneimine.

infected better than lipofectin and naked PLL methods [164]. Complex of PEI–PEG and folate would increase transfection of siRNA coding plasmid and specificity toward folate receptor overexpressed cancer cells [165]. Zhuo *et al.* [166] have used 5-flurouracil attached to a cyclic core PAA dendrimer, which releases conjugates over time during hydrolysis in phosphate buffer [167]. Blending targeting and guardianship characteristics of separate systems create novel delivery applications not only with high efficacy, but also highly safe and trustable strategy.

5. Current pharmaceuticals

Beside flourishing data, more knowledge is required to decipher potentials of the RNAi pathways. On the other hand, sophisticated delivery strategies and techniques are needed to eradicate safety considerations. Despite great potential of siRNA therapy, including fast, specific (precise), economic and efficient silencing, safety issues remain as main concern. Currently, leading pharmaceutical companies are challenging toxicity and safety of the technology. It is critical to mention that not only each disease has a unique pattern, but also our knowledge of pathogenesis including related or overlapped pathways [168,169], activators and inhibitors is also vital. There have been many successful *in vivo* studies including two models of autoimmune hepatitis [170,171], hepatitis B virus [172], respiratory viruses such as influenza virus [173,174], respiratory syncytial virus [175], parainfluenza virus, sexually transmitted disease such as herpes simplex virus-2 [176]. Also, it is demonstrated that intranasal anti-SARS siRNA can decrease virus load and fever [177]. On the other hand, anti-microRNA122, an abundant liver miRNA and crucial for replication of HCV, is now successfully participating in a study along with peginterferon plus ribavirin against chronic hepatitis C viruses. miR-122 is stable in lab temperature for few days, which makes it a proper traceable diagnostic or therapeutic applications [178]. Bitko *et al.* targeted surface antigen proteins of both parainfluenza virus-3 (PIV-3) and RSV in infected mice and could inhibit viral proliferation [179,180]. In another study, intratracheal administration of lipid complex secreted protein, acidic and rich in cysteine and connective growth factor siRNAs are followed by a reduction in collagen fabrication in fibrotic lung tissues of bleomycin-induced mice [181]. Also, administration of anti-tissue growth factor-β1 (TGFβ1) and chemokine (C motif) ligand (XCL1) siRNA also enhance anti-*Mycobacterium tuberculosis* effects in infected mice [182,183]. Despite all accomplished studies without any side effects, it is reported that siRNA cationic lipid (AtuFECT01) lipoplexes may create an inflammatory reaction in lungs [132]. **Table 1** demonstrates some accomplished *in vivo* studies about different types of siRNA and delivery strategies along with their target genes.

6. Conclusion

Application of siRNAs due to their highly specific activities has become extremely attractive in development of new drugs. In summary, it can be concluded that there are two major challenges in design of siRNA drugs. First, design of highly specific siRNAs to avoid cross-contamination and nonspecific silencing effects. Second, designed siRNAs should maintain efficient structure and stability during local or systemic delivery. Despite of considerable successes in structural modifications of siRNAs, there still are many challenges in cross-reactivity of designed siRNAs (nonspecific binding). Currently, one of the important challenges in siRNA bioinformatics is target prediction, which there is no proper prediction tool with certain drug design grade, still. Besides specific challenges in siRNA therapeutics (**Table 2**), a specific delivery method targeting a specific tissue or cell is another fundamental challenge.

7. Expert opinion

Investigation of RNAi is one of the milestones in modern biology. Recent RNAi advances in molecular biology and reverse genetics speeded developing novel screening tools and elucidating signaling pathways. The ability to silence a target gene in a rapid and specific manner facilitated deciphering genome-wide gene functions. Beside technical potentials, RNAi has special clinical merits. The possibility of designing gene-specific drugs, especially in genetic diseases, motivated many investigators and huge investment in RNAi therapy proposals. However, during 2011 some companies

Table 2. Abstract information about ongoing clinical trials of different siRNA products against various targets. Failed trials are not included.

NCT ID	Name	Condition	Delivery	Phase	Status	Target
NCT00557791	Bevasiranib & Lucentis	Wet AMD	Naked siRNA	I	Withdrawn	VEGF
NCT00257647	SV40 siRNA Vectors	CML	Pseudoviral particles	I	Completed	Unknown
NCT00306904	Bevasiranib	Diabetic macular edema and degeneration	Naked siRNA	II	Completed	VEGF
NCT00554359	I5NP	Kidney injury	Naked siRNA	I	Completed	p53
NCT00658086	ALN–RSV01	Respiratory syncytial virus infections	Naked siRNA	II	Completed	RSV nucleocapsid
NCT00689065	CALAA-01	Solid tumors	Cyclodextrin NP	I	Active	RRM2
NCT00716014	TD101					Pachyonychiacongenita
Naked siRNA	I	Completed	K6a (N171K mutation)			
NCT00938574	Atu027	Solid tumors	LNP	I	Completed	PKN3
NCT01064505	QPI-1007	Optic atrophy	Naked siRNA	I	Completed	CASP2
NCT01158079	ALN–VSP02	Solid tumors	LNP	I	Completed	KSP and VEGF
NCT01262235	TKM–080301	Cancer	LNP	I	Recruiting	PLK1
NCT01437059	ALN–PCS02	Hypercholesterolemia	LNP	I	Completed	PCSK9
NCT01445899	PF-655 (PF-04523655)	Choroidal neovascularization	Naked siRNA	II	Active	RTP801 (Proprietary target)
NCT01518881	TKM–100201	Ebola-virus infection	LNP	I	Recruiting	VP24, VP35, Zaire Ebola L-polymerase
NCT01591356	siRNA–EphA2–DOPC	Advanced cancers	LNP	I	Recruiting	EphA2
NCT01617967	ALN–TTR02	Transthyretin-mediated amyloidosis	LNP	II	Recruiting	TTR
NCT01676259	siG12D LODER	Pancreatic cancer	LODER	II	Recruiting	KRAS
NCT01739244	SYL040012	Ocular hypertension	Naked siRNA	II	Recruiting	ADRB2
NCT01776658	SYL1001	Ocular pain	Naked siRNA	I, II	Recruiting	TRPV1
NCT01780077	RXi-109	Cicatrix scar prevention	Self-delivering	I	Recruiting	Connective growth factor
NCT01814839	ALN–TTRsc	Transthyretin-mediated amyloidosis	siRNA–GalNA	I	Recruiting	TTR
NCT01872065	ARC-520	HBV	DPC	I	Recruiting	Conserved regions of HBV

HBV: Hepatitis B virus.

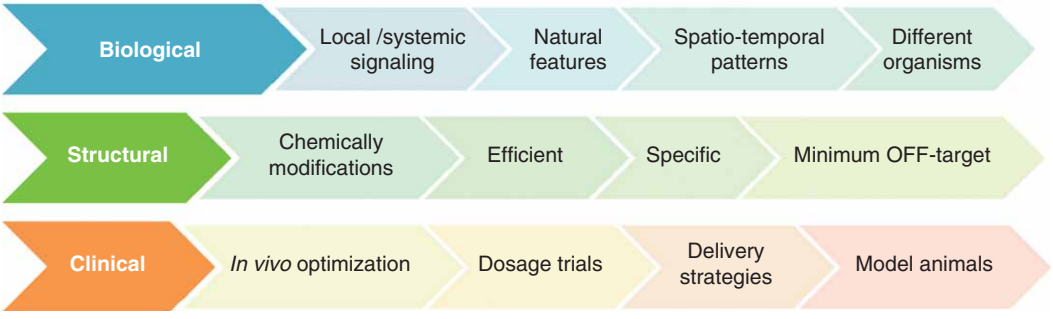

Figure 6. Various challenging areas of siRNA therapy from synthesis to post-delivery.

announced their dissuasion from developing therapeutic siRNAs.

Generally, several stepwise settings 'from the bed to the bedside' should be considered in siRNA therapy, including biological, structural, *in vitro*, *in vivo* and clinical settings (**Figure 6**). Incognito local and systemic signaling pathway of siRNAs is one of the weak sides of RNAi-based therapy. The mechanism that circulatory siRNAs transfer signals between cells is not understood well. Therefore, biological finding in local/systemic signaling of siRNAs can improve

their *in vitro* and *in vivo* applications. However, RNAi is a conserved phenomenon in many organisms, but there are some differences, too. For example siRNA–mRNA binding features are different in animals and plants but both obey complementarity roles. Therefore, many basic researches are demanding to uncover their natural features and signaling in an organism-specific method, which is time-consuming and needs financial supports (biological settings).

We know major structural features in silencing target mRNAs but there are still many unknown issues. Bioinformatics studies have revealed some structural and sequential conservations in siRNAs, but multifarious kinds of small noncoding RNAs and their diverse features is one of the challenges to develop target-specific, efficient and stable structures (structural settings).

Certainly, the challenges are not only dedicated to biological and structural challenges. *In vitro* optimization of siRNA pharmacokinetics and bio-distribution is a key step. Cell culture and tissue culture methods that mimic 3D structure and cellular composition of body facilitate this step. Checking possible off-target effects, delivery efficacy and dosage are some important factors in this step and off-target effects are not easy to investigate within a cell/body (*in vitro* settings).

Transmission of *in vitro* findings to *in vivo* systems faces with new challenges, which is the principal cause reason of dissuasion of big companies from RNAi therapeutics. Systemic delivery of drugs in a cell- and gene-specific manner is a knotty problem (*in vivo* settings). Technically, it is impossible to screen all body cells for possible cross-reactivity. Systemic distribution of siRNA is risky when we don't have a secure targeted delivery strategy and we don't have any strategy to prove the claim. Unexpected side effects of systemic delivery of siRNAs are a factual hindrance.

Anatomical barriers, drug stability and availability, immunoreactivity and existence of various delivery routes, different genetic backgrounds are major clinical challenges. According to the trend of recent failed and succeeded clinical trials, it is expected that siRNA drugs based on local administrations (such as solid tumors, inflammation, local and organic viruses such as the influenza) during foreseeable features. But, systemic application of siRNAs (for systemic diseases) will be delayed until the resolution of the gap of targeted drug delivery. Therefore, every success in targeted delivery of drugs will be applied in development of siRNA delivery strategies.

Acknowledgment

Authors thank Prof. Amir Maousavi (National Institute of Genetic Engineering and Biotechnology; NIGEB) for his encouragement and critical comments.

Declaration of interest

The authors were supported by Chemical Injuries Research Center, Baqiyatallah University of Medical Sciences,Tehran Iran. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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