Invited Review

siRNA: Sojourn from Discovery to Delivery Challenges and Clinics

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ABSTRACT

Since the Nobel prize-winning discovery of RNA interference (RNAi) in 1998, considerable money has been invested in the investigation of therapeutic potential of RNA interference (RNAi) and its application in human diseases. Encouraging results of ongoing clinical trials for the treatment of age-related macular degeneration and respiratory syncytial virus have further propelled the investment, research, and hope for a promising outcome. Due to the astonishing efforts in this field, more than 13 siRNA-based formulations are now being evaluated in clinical trials, more than 80 patents have been granted, and more than 500 patent applications have been published in 2009 in the United States alone. Despite this brisk growth, the application of RNAi therapeutics requires the development of safe, effective, and clinically suitable delivery carriers. This review highlights the evolution of siRNA technology, need of siRNA in therapeutics with specific focus on delivery challenges and clinical status, and an overview of its patent thrust.

Introduction

A journey on track since 1990, starting from the observation of pigmentation downregulation in petunia flowers by Napoli and Jorgensen, through the explanation of double-stranded RNA (dsRNA) as triggers for RNAi in 1998 by Fire and Mello and further efforts for chemical synthesis of dsRNA by Tuschl and colleagues in 2001, is about to cross the extension of therapeutic opportunities to arrive at the destination of commercialization [Figure 1].[1-4] A far-reaching research done on animals for applying RNAi in diseases like cancer, viral infections, age-related macular degeneration and rare diseases like pachyonychia congenita has moved siRNA forward in clinical trials for many local and systemic therapeutic applications.[4]

Manifestation of various diseases is through the expression of a particular disease-causing protein produced due to the viral infection and/or imbalanced expression through faulty genetic information, and siRNA can be designed to block the production of any disease-causing protein before its actual formation, by degrading its complementary mRNA transcript. Thus, a combined knowledge of siRNA designed therapy and human gene sequence can practicably target almost any impossible disease.[4-5] Hence its comprehended prophylactic potential should be well deciphered with high propensity to ensure its utilization and availability in the deadly epidemic conditions like swine flu, more recently. Also, this can actuate the still quiescent potential of siRNA as personalized therapy for many individual specific diseases like cancer and mutating viral diseases. In the past few years, this technology has been acknowledged by researchers for commercial interest to account its therapeutic potential as a novel, quick, potent, highly specific, and cost-effective way of controlling diseases by knocking down the expression of specific genes.

Before the identification of siRNA as an antisense technology, other antisense molecules like oligonucleotides, ribozymes, and aptamers have previously been studied, out of which one antisense oligonucleotide Vitravene™ (Fomivirsen) and one aptamer Macugen™ (Pegatinib) are enjoying an FDA-approved status.[6] Encouraged from these clinically approved antisense technologies, siRNA is now being evaluated for almost all diseases and more than 13 products have entered clinical trials [Table 1].[111] Even after encouraging results from preclinical and clinical studies, none of the systemically administered antisense agents has entered the market till date. Many unresolved challenges of targeting, potency, duration of effect, specificity, and safety still need to be overcome to destine a dream of commercialization.

Overwhelming efforts have already been made and are still in progress for the better development of siRNA therapeutics vitally via appropriate chemical modifications and embodiment in suitable delivery agents. Today, the trend of industrial alliances with proficient siRNA market players for licensing their proprietary platform technologies has afforded a firm grip to this technology. Furthermore, the gravity of patents granted and the figure of pending patent applications, just in 2009, reveals a marvelous

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Table 1: Current clinical status of siRNA therapeutics

Product details	Company and strategic alliances	Drug target/tissue	Indication	Type and route of delivery	Status
siRNA Cand5/ bevasiranib	Acuity/later licensed by Opko	VEGF/eye	Age-related macular degeneration (AMD)	Naked siRNA, intravitreal injections	Terminated at Phase III
		VEGF/eye	Diabetic retinopathy	Naked siRNA, intravitreal injections	
Sirna-027/now AGN-745	Sirna Therapeutics/later acquired by Allergan	VEGF/eye	AMD	Naked siRNA, intravitreal injections	Terminated at Phase II
RTP-801i	Silence Therapeutics (formerly Atugen),	VEGF/eye	AMD	Naked siRNA, intravitreal injections	Phase II
Atu027	AtuRNAi technology sublicensed to Pfizer via Quark's license	Targets the PKN3 molecule in cancer cells	Cancer	siRNA incorporated in AtuPLEX delivery platform, intravenous	Phase I
AKIi-5		P53 gene/kidney	Acute kidney injury in kidney transplantation	Chemically modified siRNA with AtuRNAi technology, intravenous	Phase I/II
DGFi			Delayed graft function in kidney transplantation		Phase I/II
ALN-RSV01	Alnylam Pharmaceuticals	RSV nucleocapsid/lungs	Respiratory syncytial virus (RSV) infection	Naked siRNA, intranasal	Phase III
ALN-VSP	Alnylam Pharmaceuticals	Kinesin spindle protein (KSP) and VEGF/liver	Liver cancer	Two siRNA molecules formulated in lipid nanoparticles, intravenous	Phase I
NUC B1000	Nucleonics	4 HBV genes/liver	Hepatitis B antiviral agent	Plasmid DNA formulated in cationic lipid delivery system, intravenous	Phase I
TD101	TransDerm (Santa Cruz, CA, USA)	Targets the N171K mutant form of the gene/skin	Pachyonychia congenita	Two delivery methods: 1. Soluble tip microneedle array 2. Topical gene cream, lipid-based technology	Phase Ib
CALAA-01	Calando Pharmaceuticals	M2 subunit of ribonucleotide reductase/solid tumors	Anticancer	RONDEL (RNAi/ oligonucleotide nanoparticle delivery/intravenous	Phase I
MDR-03030	MDRNA Inc.	Targets the conserved region of the influenza viral genome	Acts on influenza viral genome with its ability to mutate around the compound	Combined UsiRNAs with DiLA2 delivery platform, intranasal	Preclinical phase
rHIV7-shI-TAR-CCR5RZ	Benitec	HIV tat/rev gene, TAT responsive elements, CCR5 receptors/targets stem cells	AIDS-related lymphoma	DNA-based plasmid expressing anti-HIV RNA, systemic	Phase I

growth presently and forthcoming blessing in therapeutics and marketing. This review highlights the above summarized aspects of siRNA, its need in therapeutics, and key challenges in its targeted delivery with anticipated successful clinical applications and patentability.

RNA interference (RNAi): An overview

RNAi is a posttranscriptional gene silencing (PTGS) phenomenon that downregulates the expression of a specific protein by degrading the specific mRNA which carries the message for translation of that particular protein. It acts as a natural defense mechanism against various viral attacks in the body. In mammalian cells, the introduction of long dsRNA (>30 nt) initiates a potent antiviral response, exemplified by nonspecific inhibition of protein synthesis and RNA degradation. The mammalian antiviral response can be bypassed, however, by the introduction or expression of siRNAs. In 2001, Tuschl *et al*. reported that chemically synthesized 21 base-pair double-stranded RNA molecules suppressed the target gene with high specificity.[3] Since then, chemically synthesized siRNA has been widely used for biological researches to identify gene functions.

Mechanism of RNAi

The mechanism by which RNAi is thought to work is a very complicated one. The process by which RNAi occurs in different organisms varies slightly because of different proteins used in the process of RNAi. What triggers RNAi or how does a particular mRNA doesn't translate its message into specified protein? Yes, such interference from the translation process needs involvement of some triggers that otherwise are not contacted during protein formation. Long double-stranded RNAs (dsRNAs) are the well-established triggers to silence the expression of target genes in a variety of organisms and cell types (e.g., worms, fruit flies, and plants). Upon introduction, the long dsRNAs enter a cellular pathway that is commonly referred to as the RNA interference (RNAi) pathway. This

process proceeds through various sequences of steps [Figure 2]:[7]

1. First in the initiation step, the dsRNAs, introduced by viral infection or after its chemical synthesis, get processed into 20-25 nucleotide RNAs, called small interfering RNAs (siRNAs), by an RNase III-like enzyme called dicer.
2. Then, the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs).
3. While getting in the process of assembly with RISCs, siRNAs undergo unwinding with the help of helicase enzymes. This activates the complex leaving only the antisense strand associated with the RISC. Other strand of the duplex called sense or passenger strand gets degraded by the enzymes in the cytoplasm. The strand selection depends on the relative thermodynamic stabilities of the two duplex ends, and it is least stable at the 5′ end of the duplex that is recognized and asymmetrically unwound by the Piwi-Argonaute-Zwille (PAZ) domain of argonaute 2, a multifunctional protein within the RISC.
4. The activated RISC guides the siRNA toward its complementary mRNA.
5. After binding of siRNA with complementary sequence-specific mRNA, Argonaute enzymes associated with the RISC complex cleave the target mRNA nearly from the middle of the region bound by the siRNA strand.
6. This cleaved mRNA is recognized by the enzymes which destroy the cognate RNA. Hence it blocks the expression of a protein particular to the degraded mRNA.
7. The activated RISC complex can propagate to destroy additional mRNA targets, which further amplifies siRNA-induced gene silencing. This carrying capability ensures a therapeutic effect for 3-7 days in rapidly dividing cells, and for several weeks in nondividing cells. Finally, siRNAs are diluted below a certain therapeutic threshold or degraded within the cell, and so repeated administration is necessary to achieve a persistent effect.

Why siRNA therapeutics

The possibility of chemical synthesis and its amenability to chemical modification opens up enormous possibilities for using siRNAs as potential drug candidates. Their appropriate chemical modification and design can reduce off target effects and make them suitable for particular application with minimum toxicity. Many key features of siRNAs have attracted worldwide attention toward their therapeutic use. Most of the drugs have limited targets and rely on symptomatic relief of the disease after the formation of a disease-causing protein, whereas siRNA can efficiently control the disease by blocking the expression of disease-causing protein before it is formed, thus treating the root cause of a disease. It can be successfully developed as a prophylactic agent for already known epidemic diseases where the disease-causing elements have already been established and can be targeted through designed disease-specific siRNA before the disease contraction in epidemic areas. Drugs like monoclonal antibodies also have limited targets and act mainly on circulating proteins or cell-surface receptors. siRNA is a potent highly specific therapeutic agent that can be designed to access unlimited number of intracellular targets with the knowledge of sequenced human genome and delivery. Further, certain diseases may be caused by the mutation in a single allele, in which case a specific siRNA can target the disease-causing mutation leaving the normal allele intact.[8] Talking about other antisense technologies like antisense oligonucleotides, these are short lived since they cannot be stably retained in specific gene silencing complexes. Therapy with these antisense oligonucleotides relies on achieving oligonucleotides concentration in nucleus and their nuclear access in the target tissue which requires higher dose and repeated administration. On the other hand, antisense strand of siRNA acts in the cytosol where it gets stably incorporated into the RISC silencing complex to achieve prolonged gene silencing even in a very small concentration.[9] Furthermore, most of the viral diseases are the manifestation of changing viral mutations that limits the use of a particular antiviral drug that acts for that particular mutant only. A cocktail of more than one mutant-specific siRNA in a single delivery carrier is a wonderful remedy for mutating viral diseases and cancer due to more than one gene mutation.[8-13] One such formulation, developed by Alnylam Pharmaceuticals, having two siRNA molecules formulated in lipid nanoparticles for the treatment of liver cancer is under phase- I clinical trials [Table 1]. Besides this, multidrug resistance is a tough aspect in many chemotherapeutic and antibiotic therapies. Since siRNA can specifically be targeted to combat the expression of resistance-causing proteins, its potential is now being evaluated as a chemosensitizing agent to enhance the effect of following chemotherapy. Studies have been performed for the pretreatment of target tumor xenografts with siRNAs or short hairpin RNA (shRNA)-encoding plasmids to knock down a multidrug resistance protein and subsequent treatment with targeted minicells containing cytotoxic drugs could eradicate previously drug-resistant tumors.[14] Moreover, certain diseases like cancer are patient specific caused due to some specific gene defect of mutation. siRNA potential should be explored as personalized therapy by designing it complementary to the target mRNA of individual patients.[8] All the above facts reveal the wonderful position of siRNA therapeutics to overcome dose-limiting toxicity of chemotherapeutics, heterogeneity and drug resistance of cancer cells, and difficulties of targeted delivery to tumors. It has been explored for administration through various routes. Its potential through pulmonary, mucosal membranes like intravaginal, ocular, and nasal, and other localized and systemic delivery routes has been explored extensively.[15-20] Recently, a study unfolded the potential of siRNA through the oral route for targeting macrophages. Results from the study demonstrate that gene silencing using Map4k4- siRNA-loaded particles is a potent method for targeting the macrophage proinflammatory mediator TNF.[21] All the above aspects have attracted the interest of many researchers but one fact that is still in its infancy is its use as personalized therapy so as to benefit many individuals suffering from particular gene defects. This strategy requires the attention of research scientists for its best utilization to make it sound on therapeutic grounds. Above all, the achievement of approved siRNA clinical applications requires an understanding of the barriers to siRNA delivery, the effective design of siRNA, and its combination with an appropriate delivery system.

Barriers to siRNA-targeted knockdown

The ultimate goal of siRNA therapeutics is the treatment of some diseases associated with a particular part or the system of the body. To elicit its action, siRNA should reach the disease-specific site for which it has to cross many barriers. To invade a particular cell, siRNA can either be delivered locally or systemically. For local delivery there are few techniques like hydrodynamic iv injection

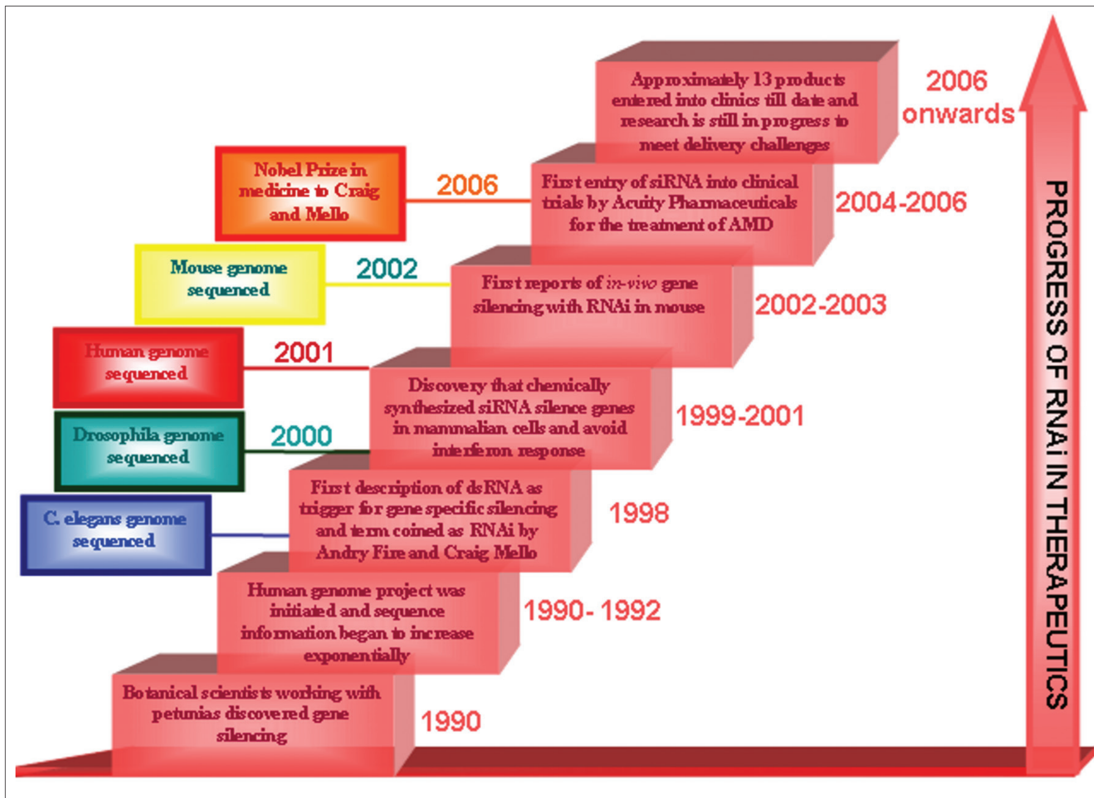

Figure 1: Major events during the understanding, development, and application of RNA interference (RNAi) in therapeutics

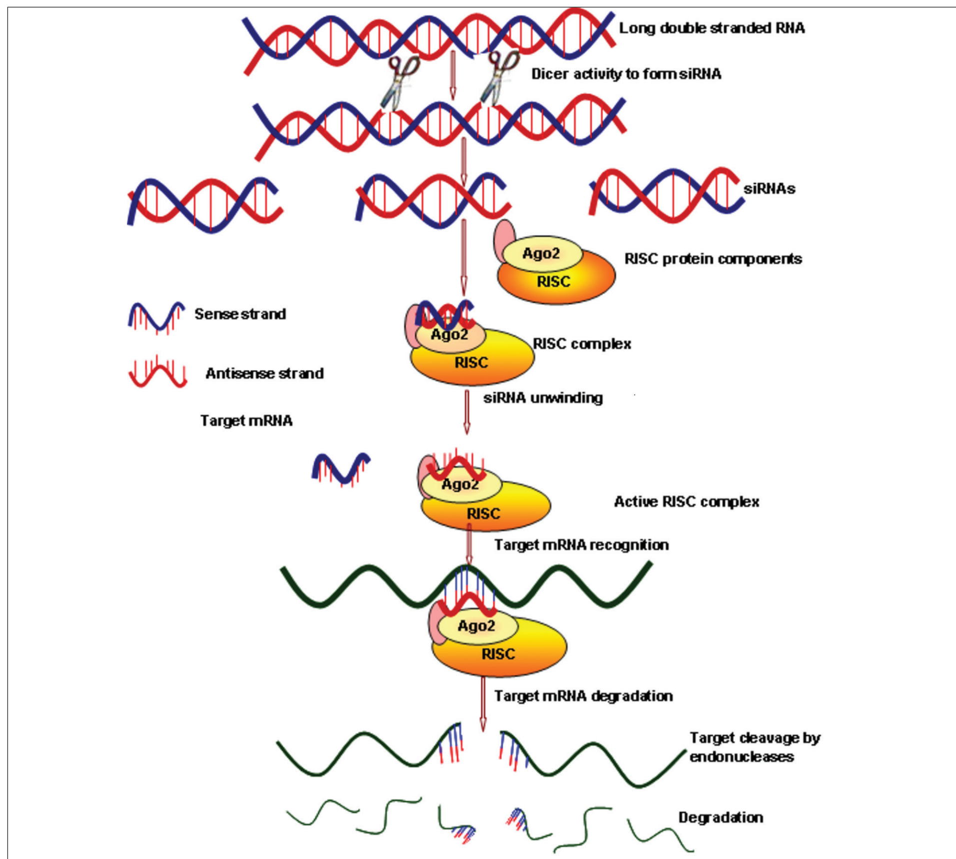

Figure 2: Basic steps involved in the mechanism of RNAi

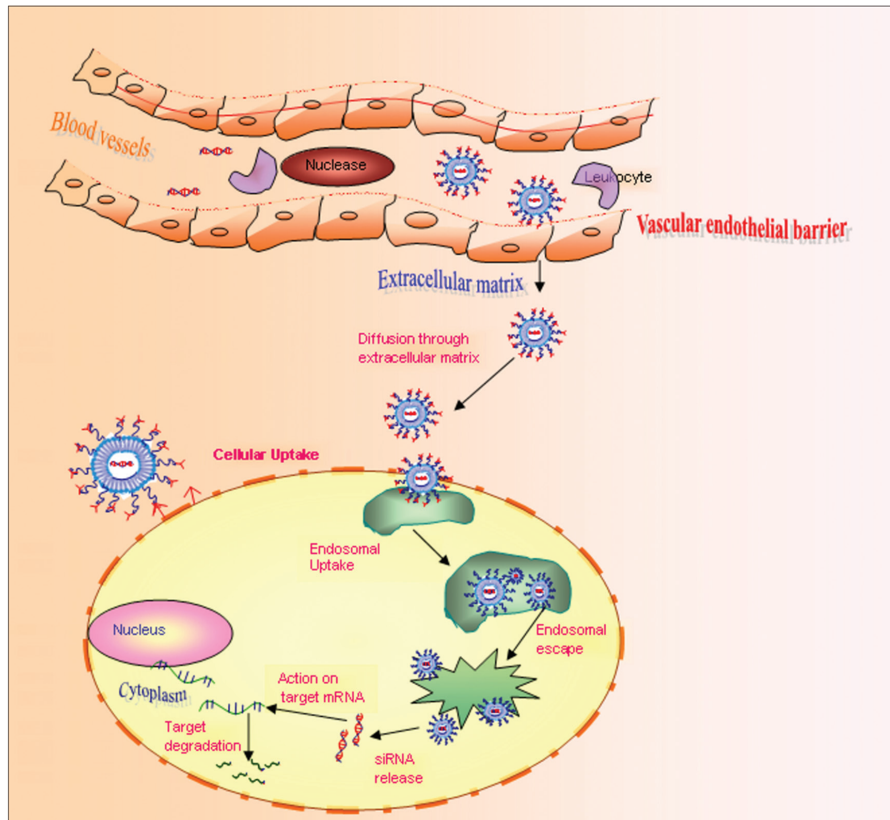

Figure 3: Major physiological barriers to the systemic siRNA delivery

and electroporation which have their own shortcomings of large injection volume and cytotoxicity, respectively. For many therapeutic applications, siRNA has to be delivered systemically.[22-25] After systemic injection, siRNA encounters many challenges that need to be handled to extract maximum therapeutic potential of this molecule. siRNA as such is polyanionic (40 negative phosphate charges) in nature and has a molecular weight of 13 kDa which makes it difficult to cross various cell membranes while in a delivery carrier. Thus major attention of the research is toward the effective design of siRNA through chemical modification and/or its combination with an appropriate delivery system to attain better targeted delivery to the cells and tissues. Here we will focus on the major barriers to siRNA delivery [Figure 3] and ways to handle this issue for an efficient delivery.

Barrier of nuclease enzymes

While crossing plasma and tissue after injection, siRNA encounters nuclease enzymes which cause 3′ end and internucleotide bond cleavage making double-stranded siRNA short lived. But many successful efforts to overcome this barrier have been made over the years, specifically for antisense oligonucleotides that have been studied in siRNA too.[26] The study of biochemical properties of siRNA has revealed the possibility of remarkable modifications at the 2′-OH position of pentose sugars and 3′ half of the siRNA structure. The most common stabilizing modification is the substitution of sulfur for oxygen to form phosphorothioate siRNA, but has a disadvantage of toxicity due to nonspecific binding to various proteins. Further, 2′-OH modifications, locked nucleic acids (LNAs), peptide nucleic acids (PNAs), morpholino compounds, and hexitol nucleic acids (HNAs) are few strategies to improve mRNA affinity as well as stability toward nucleases. Moreover, the judicial inclusion of LNAs, 6 carbon sugars instead of ribose, 2-F and 2-OMe modifications and the use of gapmers have shown improved existence in biological milieu leading to a prolonged pharmacological action.[27] The use of siRNA conjugates and siRNA delivery carriers for bloodstream stability is also on a great move and would be discussed later. Out of the all above-discussed modifications, none alone can be considered as the best solution to overcome nucleases. Rather depending upon the target tissue, desired action, and other components of the delivery system, one should evaluate and decide the suitable modification to be applied.

Glomerular filtration and RES uptake

Molecules less than 70 kDa and 5 nm in diameter undergo glomerular filtration. siRNA is unable to avoid this step due to its much lesser molecular weight of 13 kDa and thus get excreted soon after its administration.[28-30] However, the size of siRNA can be designed by its complexation with or incorporation into suitable carriers. Body's reticuloendothelial system (RES) system works in the defense of the body against foreign pathogens, to remove cellular debris, and clear cells that have undergone apoptosis with the help of its soldiers: the phagocytes, which include monocytes and macrophages. Due to the abundance of RES components in liver and spleen cells, siRNA gets accumulated in these organs after systemic administration.[31,32] Thus this feature is useful while knocking down target molecules in these tissues, but on the other hand causes toxicity due to long-term deposition in RES organs. Particles smaller than 100 nm tend to leak out from the intercellular junction of the

Table 2: Suppliers for gene-specific siRNA and RNAi kits

Company	Internet site	Products and services	Application
Allele Biotechnology	www.allelebiotech.com	LineSilence RNAi Kit	The kit uses the allele linear-cassette technology and eukaryotic RNA polymerase III promoters to prompt the cells to produce their own dsRNAs endogenously
Ambion	http://www.ambion.com	Silencer Express	Silencer range of kits, vectors, siRNAs, and reagents for RNAi; kits and products for RNA synthesis, isolation, quantitation, and analysis
		MEGAscript RNAi Kit	
		pSilencer adeno	
		Custom siRNA Synthesis	
Atugen AG (now Silence Therapeutics)	http://www.silence-therapeutics.com	AtuRNAi	siRNA-based drug discovery; custom phosphorothioate antisense RNAs and siRNAs for drug target identification and validation
BD Biosciences	www.bdbiosciences.com	BD Knockout	Provides transient and stable expression of siRNA constructs in dividing, nondividing, and hard-to-transfect cells and plasmid-based vectors with options for adenoviral and retroviral delivery
		RNAi Systems	
Cenix BioScience	http://www.cenix-bioscience.com	Lipofection reagents (including beta testing of various prerelease reagents) emerging	Genome-based high-throughput applications of RNA interference for assay and drug action in human cells; target discovery and validation by RNAi screening and profiling
		HT-electroporation approaches	
Dharmacon	http://www.dharmacon.com	siSTARTER b-Gal/luciferase kit for RNAi	Validated siRNA with three levels of silencing capabilities that can occur based on target site selection; siRNA kits and arrays; custom RNAs; large-scale RNA synthesis
		Duet library of siRNA, microRNA, shRNA, RNAi controls, transfection and ancillary reagents	
		Custom synthesis and contract research	
Eurogentec	http://www.eurogentec.be	siRNA design custom	Custom siRNA for RNAi and various siRNA transfection and expression assay reagents
		siRNA ICAFectin442	
		siRNA transfection reagent	
		siRNA positive and negative controls	
Genlantis	www.genlantis.com	GeneSilencer	GeneSilencer siRNA transfection reagent is a novel cationic lipid formulation specifically designed
Imgenex	http://www.imgenex.com	PSuppressorAdenoRNAi vector	For efficient delivery of siRNAs (small interfering RNAs) into a wide variety of cell types particularly those not amenable to efficient transfection
Invitrogen	www.invitrogen.com	Block-iT Complete Dicer RNAi kit	siRNA cloning vectors; custom siRNA in plasmid and viral vectors
		Block-iT Lentiviral RNAi expression system	
		Block-iT RNAi TOPO transcription kit	
InvivoGen	http://www.invivogen.com	psiRNATM system	siRNA cloning vectors; custom siRNA in plasmid and viral vectors
Mirus Bio Corp.	http://www.genetransfer.com	TransIT transfection reagents for siRNA and vectors	Unique lipid and polymer formulations to achieve superior delivery with minimum cytotoxicity and reagent kits required for the preparation and transfection of siRNA expression cassettes
		siXpress Human H1 PCR vector system	

Table 2: (Contd...) Suppliers for gene-specific siRNA and RNAi kits

New England Biolabs	http://www.neb.com	HiScribe RNAi transcription kit ShortCut RNAi Kit	High-yield synthesis of double-stranded RNA. Reagents for the synthesis, processing, and transfection of dsRNAs for in vitro transcription
Novagen	http://www.novagen.com	RiboJuice siRNA transfection reagent	Efficient delivery of siRNA for targeted gene suppression
OligoEngine	http://www.oligoengine.com	pSuper RNAi RNAi duplexes RNAi precursors Micro RNA repressors	pSUPER RNAi vector system for gene silencing; custom oligonucleotides for all applications and expert RNAi design
Promega	http://www.promega.com	psiCHECK™-1 and psiCHECK™-2 Vectors Bottom of Form GeneClip™ U1 Hairpin Cloning System—Basic Bottom of Form MicroRNA Biosensors: Application for the psiCHECK™-2 Vector Functional siRNAs and Hairpin siRNAs using the T7 RiboMAX™ Express RNAi System	Vectors, reagents and kits for genomics, proteomics, and cellular analysis; fast and easy cloning of human target sequences; kits for siRNA production and delivery
Proligo	http://www.proligo.com	Custom siRNA synthesis	Custom and speciality DNA and RNA oligos; siRNA oligos for RNAi
QIAGEN	http://www.qiagen.com	4-for-Silencing siRNA duplexes Custom siRNA synthesis	Custom siRNAs and transfection reagents; cancer siRNA oligo set, version 1.0, pool of four siRNA duplexes to ensure at least one duplex providing 70% knockdown of gene
Roche Applied Science	www.roche-applied-science.com/sis/fugene/	X-tremeGENE siRNA - transfection reagent FuGENE 6 transfection reagent	Production of significant protein expression and effective knockdown levels. Highly effective nonliposomal formulation tool for transfecting DNA into eukaryotic cells
Sequitur	http://www.sequiturinc.com	Stealth RNAi synthesis	Antisense and siRNA compounds
Spring Bioscience	http://www.springbio.com	Knock-down siRNA Kit	Knockdown kits for producing siRNAs from target DNA sequence; products for gene cloning, target screening, and protein expression analysis
Stratagene	www.stratagene.com	GeneEraser Suppression-test system	Easy, rapid system for identifying effective siRNAs or shRNAs. Luciferase expression or fluorescent protein is read out for suppression
Upstate Group Inc.	www.upstate.com	siImporter	Cationic lipid formulation designed for the transfection of siRNA and plasmid DNA into a wide variety of mammalian cell lines

capillary endothelium (70-100 nm pores) and to the interstitial space of hepatic sinusoid and get trapped by hepatic parenchyma cells or Kupffer cells. On the other hand, particles greater than 200 nm are taken up by RES such as liver and spleen, and are rapidly removed from systemic circulation. Thus the suitable size is around 100 nm for the siRNA delivery carrier. Tagging of negatively charged siRNA with cationic carriers can improve its stability in the bloodstream. But these cationic carriers react with negatively charged molecules such as serum proteins, platelets, and RBCs in the bloodstream and form undesirable aggregates. It was shown that reduced RES trapping was achieved by the surface modification of cationic carriers with polyethyleneglycol (PEG), dextran, or polyglycerol.[29-32] Understanding the pharmacokinetics and biodistribution of siRNA and its interaction with the biochemical machinery of the specific tissue can help to design best-suited siRNA delivery carriers.

Endothelial lining

Endothelial lining has two main routes of transport: one paracellular, a small pore system which is high in abundance and another is transcellular, a large pore system which is low in abundance. siRNA is too large (13 kDa) and too negatively charged to cross cellular membranes. Some specialized tissues like those of liver and spleen and certain tumor cells allow the access of larger molecules due to the presence of large fenestrations in their

endothelium. The endothelial cell line presents an opportunity as a site of action for siRNA in treating diseases like tumor angiogenesis, control of blood pressure, and regulation of hemostasis.[33-35] However, it acts as a major barrier for the therapy of various diseases requiring the action of siRNA inside the cell where it needs to cross the endothelial lining. The apparent hydrodynamic radii of highly charged polynucleotides like siRNA are larger than those of either globular proteins or random coil uncharged polymers (e.g., dextrans) of a similar molecular mass. Thus the tissue permeation rate would be slower relative to that predicted on the basis of molecular weight. An egress of the siRNA through endothelial lining can be made possible through molecular conjugates and targeting ligands or with "cell penetrating peptides."[9]

Cellular uptake and subcellular distribution

siRNAs function by translation arrest by approaching target mRNA in the cytoplasm. For gene silencing effect, siRNA must undergo cellular uptake. The main mechanism for the cellular capture is receptor-mediated endocytosis.[36] This can be achieved by tagging siRNA with a receptor-specific ligand for the target tissue. Alternatively, membrane permeable peptides (penetratin and transportan) may be coupled to siRNA to facilitate their intracellular uptake.[37,38] After internalization, there occurs sequential intracellular trafficking into a variety of low-pH endomembrane compartments, including early/sorting endosomes, late endosomes/multivesicular bodies, and lysosomes. Finally, siRNA must exit from the endosome to reach target mRNA in the cytoplasm. This can be achieved by conjugating siRNA with an endosomal release signal peptide linked to a nanocarrier.[40] The elucidation of the cellular uptake, vesicular trafficking, and endosomal release pathways of oligonucleotides can help optimize the chemistry of nucleic acid drugs for greater biological effectiveness.[40,41]

Thus siRNA delivery has many key challenges as per its path to reach the target, including the fight with nucleases and RES, crossing endothelial lining, cellular capture and finally endosomal release into the cytoplasm for binding to its cognate mRNA for gene silencing. Unmodified, naked siRNAs are relatively unstable in blood and serum, and have short half-lives *in vivo*. Suitable chemical modifications to the backbone, base, or sugar of the RNA can be introduced into the RNA duplex structure so as to enhance biological stability without adversely affecting the gene-silencing activity. While "naked," chemically modified siRNA has shown efficacy in certain physiological settings such as the brain and the lung, there are many tissues in the body that require an additional delivery system to facilitate transfection. Thus, we will next review the numerous efforts made in the effective design of siRNA and its formulation with a delivery system that not only enhances cellular uptake but also affords biological stability for the gene-silencing activity sustained for several days.

Effective design of siRNA

siRNA should be designed so as to make it chemically stable and with high specificity for the sequence complementary to the mRNA target. Computer softwares for specific siRNA designs are now available that have incorporated as many selection rules as possible from the accumulating knowledge of RNA interference. Softwares can be downloaded by researchers and can be used to design effective siRNA sequences more easily. Rational siRNA design schemes are being developed that are based on an understanding of RNAi biochemistry and on naturally occurring miRNA (micro-RNA) function.[42,43] Presently, custom siRNA synthesis service is available through a number of companies [Table 2], such as Allele, Ambion, Dharmacon, and QIAGEN. While there are currently no reliable methods to identify the ideal sequence for a siRNA, a number of parameters [44, 45] that are to be taken care of are:

1. Sequence region: selection of a target cDNA region, 50-100 nucleotides downstream of the "ATG" start codon
2. Selection of a 5′-AA (N19) UU target mRNA sequence where N is any nucleotide
3. Sequences with repeats of three or more Gs or Cs are avoided as their presence initiates intramolecular secondary structures preventing effective siRNA silencing hybridization. As an option, repeat stretches of As and Ts can also be eliminated, as they tend to reduce the specificity of the target sequence
4. G-C content of the target site: 50% G/C content in the target sequence is preferred. Sequences with a low GC content (<60%) have a better chance to be functional siRNA sites than those with a high GC content (>60%).
5. Avoidance of 5′ or 3′ untranslated regions and high G-rich areas
6. Confirmation of exclusive target-specific sequences
7. Off-target effects: an off-target effect is the silencing of an unintended target gene. This effect can arise due to
   a. partial sequence homology, especially within the 3′-UTR, exists with mRNAs other than the intended target mRNA and
   b. stimulation of the innate immune response. siRNA duplexes housing certain GU-rich sequences (e.g., UGUGU) can induce IFN-α, IL-6, and TNF-α via interaction with TLRs.

This effect can be reduced by the incorporation of 2′-*O*-methyl modifications into the sugar structure of selected nucleotides within both the sense and antisense strands. Additionally, screening the designed siRNA for homology with available sequence databases can minimize many off-target effects while maximizing the prediction accuracy of RNAi. But still not all immune stimulatory RNA motifs have been identified as yet; thus, empirical testing in the target system is still necessary for identifying specific and potent siRNA design.

Delivery techniques

The success of siRNA therapeutics relies on the efficient delivery to the desired site for target mRNA degradation. From the above discussion, we have got acquainted with the various hurdles to siRNA delivery and how these can be overcome to some extent through chemical modification. Although chemical modifications are effective, yet modulating the tissue distribution profiles of siRNA with chemical modifications only seem more difficult. An efficient delivery carrier is must to facilitate siRNA access to its intracellular sites of action. Currently various viral vector-based and many nonviral delivery techniques have been studied extensively for this purpose. In this review, we will concentrate, in detail, on nonviral delivery techniques. Before that, let us have a brief overview of vector-based siRNA delivery.

Vector-based delivery

These vector-based strategies involve either DNA or viral vector-mediated RNAi. In the first approach, RNA polymerase 2 or 3

promoters have been incorporated into DNA vectors along with siRNA expression cassettes (SECs). SECs are PCR products that upon transcription form RNA hairpin loop and get converted to siRNA intracellularly. RNA polymerase 2 promoters generate shRNAs that permit easier adaptation of inducible/repressible tissue- or cell-specific siRNA expression.[46] Since plasmid-based siRNA expression has limitations in cases where transfection efficiency is low, viral vectors have also been developed. Adenoviral vectors [47,48] and various retroviral vectors[49,50] (e.g., lentivirus based) are proven effective delivery systems for numerous cell types including noncycling cells, stem cells, and zygotes. Successful tranduction of P53-targeted siRNA using retroviral vectors has been shown in cell lines as well as fibroblasts. Another virus, adenovirus, has also shown favorable results in reducing cellular aggregation induced by pathological polyglutamine in a murine model. These viral vectors have a drawback of cell proliferation-dependent incorporation. But lentiviral vectors could be efficiently incorporated into nondividing cells. Lentiviral-delivered siRNA has shown long-lasting (>25 days) GFP gene silencing.[51] These early studies were carried out by transfecting chemically synthesized siRNAs, more recently; dsRNAs have been expressed from stem-loop structures encoded by plasmids, retroviruses, and lentiviruses. These vectors provide a means for *in vivo* transduction of a variety of cells, including nondividing cells, and open up a range of possible therapeutic uses for RNAi. Sequences of siRNA expressed from retroviral and lentiviral vectors have been shown to silence expression effectively in a variety of primary cells including embryonic stem (ES) cells, lymphocytes, macrophages, and dendritic cells.[52-54]

Technically, viral vectors are the most powerful tool for gene transduction but show a limited loading capacity and are difficult to produce on a large scale and elicit immunogenic effects upon administration, which prevent them from repeated administration. In comparison, nonviral or synthetic vectors do not pose safety and production issues. But on the downside, transfection efficiency with nonviral techniques still remains an unresolved key challenge.[55,56] Over the past few years, tremendous efforts have been made to induce site-specific binding and uptake through various modifications in delivery carriers.

Nonviral siRNA delivery

Various strategies for delivering siRNA to specific tissue and organ systems *in vivo* via nonviral delivery techniques are summarized below:

Hydrodynamic IV injection

Rapid injection of siRNA in a large volume of a physiological buffer effectively facilitates duplex siRNA uptake in the liver probably through membrane perturbations caused by high-pressure injection. In rats, the administration of both unmodified and chemically modified VEGF-specific siRNA resulted in effective inhibition of pathological neovascularization. However, large injection volumes required and cellular toxicity due to invasiveness of this technique limit its clinical application.[57] Currently, advances in the use of a computer-controlled, catheter-guided injection device have greatly improved the precision and reproducibility of this approach.[58]

Electroporation

Electroporation is a technique for localized siRNA delivery even for the hard-to-transfect types of cells. This technique utilizes a brief but powerful electric pulse that generates heat and causes lipid molecules of the cellular lipid bilayer to reorient and undergo thermal transition. This invasive method creates localized perturbations and hydrophilic pores in the membrane. Thus along with the benefit of high transfection efficiency, this technique has limited use due to high cell mortality. However, the process parameters like voltage, length, and number of pulses can be optimized for different cell types to get maximum transfection with minimum cytotoxicity.[59-61] Golzio *et al*. showed 23-day-long silencing effect after the administration of siRNA cotransfected with a plasmid-encoding GFP in mice. Also, there are reports revealing a safe use in the CNS of rats.[61]

Carrier-mediated delivery

To reduce off-target effects and to enhance pharmacokinetic behavior, nuclease resistance, cellular uptake, target specificity and safety, siRNA has been chemically conjugated to or incorporated into a variety of bioactive molecules, lipids, polymers, peptides, and inorganic nanostructured materials. Potential sites of the siRNA structure for conjugation with minimal influence on the RNAi activity are 3- and 5-terminus of the sense strand and the 3-terminus of the antisense strand.[62-64] siRNA being negatively charged can be efficiently carried in a cationic carrier. Here, we will review the delivery techniques for siRNA via various carriers one by one.

Lipid-based siRNA delivery

siRNA may interact with lipids to form conjugates, lipoplexes by spontaneously interacting with positively charged lipids or getting enclosed in the center aqueous compartment of liposomal vesicles formed by the phospholipid bilayer. In order to extract maximum potential, the cationic lipids employed must meet few of the following criteria. Though a positive charge is required for the interaction with negatively charged nucleic acid-based drugs, the final liposomal formulation must have a near-neutral surface charge in order to avoid immediate uptake into the lymphoid tissue of the RES. Further cationic lipids used must be biodegradable to limit toxicity due to the cationic nature. Also, cationic lipids act in destabilizing biological membranes by combining with anionic lipids to induce a nonbilayer structure. Such propensities must be maximized. Finally, in order to be biologically active, the siRNA must dissociate from cationic lipids inside the cell, requiring anionic lipids to be able to compete for associated cationic lipid leaving the free active moiety to exert its biological effect. Various lipophile-siRNA conjugates include cholesterol-siRNA conjugate (chol-siRNA), conjugates with bile salts, lipids and α-tocopherol (vitamin E)-siRNA.[65-67] A significant reduction in the target protein (apoB) in the liver could be achieved after systemic administration of these conjugates without any induction of inflammatory interferons.[65] Lipoplexes due to the spontaneous formation and an unstable nature should be prepared immediately before use. siRNA-DOTAP lipoplexes have shown a sustained effect of 12 days to selectively inhibit 1-adrenoreceptor expression.[68] Few commercially available potential enhancers of siRNA transfection are Lipofectin, RNAifect, Oligofectamine, Lipofectamine, and TransIT TKO.[68-74] Moreover, PEGylated liposomes of doxorubicin are a clinically approved delivery system which has encouraged the researchers for the evaluation of PEGylated liposomes for the delivery of siRNA. The systemic administration of PEGylated cationic liposomes termed “solid nucleic acid lipid particles” could silence the *apoB* gene in mice and nonhuman primates for more than 11 days after their single

dose. Lipid-based carriers have a tendency of passive accumulation in the liver, but can also be well targeted to other organs via attachment of a targeting ligand or antibody. The attachment of transferrin receptor-specific single-chain antibody fragment effectively delivered a HER2-specific siRNA entrapped in cationic liposomes to tumor xenografts in nude mice. After cellular entry, endosomal membranes should be destabilized to aid the release of siRNA into the cytosol that can be achieved by the inclusion of fusogenic lipids such as l-α dioleoyl phosphatidyl ethanolamine (DOPE) and/or pH-sensitive peptides such as poly-histidine-lysine in siRNA delivery systems. For the successful clinical application of siRNA lipid carriers, toxicity issues associated with various cationic lipids should also be given profound consideration. Recently, natural lipid analogs of cardiolipin have been successfully used, in delivering siRNA systemically to cancer xenografts.[75]

Polymer-based siRNA delivery

Successful *in vivo* siRNA delivery requires a carrier composed of right polymers for the desired function at various steps of delivery including extracellular stage, cell targeting, cellular uptake, and endosomal release.[76] An appropriate polymer should be chosen for the desired action. siRNA can chemically bind with cationic polymers to form complexes called polyplexes. Out of the various cationic polymers, the potential of polyethyleneimine (PEI) to deliver siRNA, while protecting from enzymatic and nonenzymatic degradation, has been widely explored.[77] PEI is available as a linear or branched polymer and in many molecular weights, ranging from 1 kDa to more than 1,000 kDa. Higher molecular weight PEIs are efficient siRNA carriers but tend to be toxic whereas branched, low-molecular-weight PEIs ($<$25 kDa) are thought to be superior to linear ones as the transfection reagents. Biodegradable polyester having lower toxicity and higher transfection efficiency has been prepared by reacting 800-Da branched PEI with diacrylates.[77-79] PEI concentration-dependent downregulation ($\geq$60%) of targeted luciferase expression was seen at 8 g/mL of the biodegradable PEI with as little as 5 nM of siRNA. Also, PEG-siRNA conjugate can be formed via a reducible disulfide linkage of siRNA with poly(ethylene glycol) (PEG), a biocompatible, hydrophilic, and nonionic polymer which has shown much higher levels of siRNA stability (up to 16 h) than the naked siRNA in the presence of 50% serum. PEG can also be used as a shielding polymer when coupled with cationic polymers incorporating siRNA.[80] Reducible polymers are the carrier of choice for siRNA delivery due to its cytosol-specific degradation capabilities.[81] PEG-PLL block copolymer-based polyplexes with disulfide cross-linking stabilized the complexes extracellularly but were cleaved under the reductive conditions inside the cell. Another cationic hydrophilic polymer, chitosan, [82,83] has been explored to carry siRNA in various delivery carriers like complexes (chitoplexes), liposomes, nanoemulsions, and nanoparticles. Out of these, chitosan-modified nanoemulsions were able to transfect hard-to-transfect human lung bronchial epithelial cell-line H441 without any toxic effects and seem to have a better potential to deliver siRNA. The first polymer-based targeted delivery carrier (CALAA-01, Calando Pharmaceuticals) that got approval for clinical trials in April 2008 was the self-assembling, cyclodextrin polymer-based nanoparticles developed by Mark Davis and his colleagues. The composition of a cyclodextrin-containing polycation, PEG, human transferrin as a ligand and siRNA self-assemble into nanoparticles that could target receptors on cancer cells.[84-86] Another class of multifunctional nanoparticles for siRNA delivery was prepared based on the use of semiconductor quantum dots and proton-sponge polymer coatings. Proton-sponge layer formed by covalent grafting of tertiary amine groups on the QD surface leads to efficient siRNA release from intracellular vesicles.[87] Polymers have also been fabricated into smart micelles[88] for efficient siRNA delivery. An electrostatic interaction between siRNA and PEG-polycation block copolymers lead to the formation of self-assembled nanocarriers called polyionic complex micelles (PIC micelles). Negatively charged siRNA get completely embedded within the polyelectrolyte core due to charge neutralization by polycations. These smart polymeric micelles have a surrounded palisade of a flexible, hydrophilic PEG layer along with a ligand at its periphery for circulation stability and specific targeting with better cellular entry. pH-sensitive lactosylated PIC micelles were successfully transported to hepatoma cells with final cytosol release of hundreds of siRNA molecules on a low-pH response in endosomes.[88,89] A better strategy is to incorporate molecular units facilitating endosomal release directly into block copolymers. Hybrid nanoparticles of PEG-block poly(aspartic acid) copolymers with calcium phosphate have shown improved physiological stability. On the other hand, a polyanionic-unit of polymethacrylic acid in a block copolymer can undergo conformational changes at an endosomal pH range of 4-6 to release nanoparticles into the cytoplasm. Local and systemic administration of siRNA targeting vascular endothelial growth factor (VEGF) as PEG-siRNA/PEI PEC micelles showed localization in the solid tumor region. This property of PEC micelles may be due to the passive diffusion of nanoparticles through a leaky endothelial vascular structure of proliferative tumors, which is called the enhanced permeation and retention (EPR) effect. Besides above-discussed delivery carriers, siRNA has also been incorporated into hyaluronic acid (HA)-based nanogels.[90] HA is a natural polymer that plays important biological roles in tissue integrity, angiogenesis, wound healing, and cell motility through the interaction with receptors on cell membranes. GFP gene silencing was observed under both serum and nonserum conditions upon cotransfection of HA nanogels containing GFP siRNA with GFP plasmid/lipofectamine to HCT-116 cells. Moreover, HA has also been conjugated with quantum dots (QDs) that has shown better cellular internalization due to receptor-mediated endocytosis as compared with QDs alone. Furthermore, the PEI-HA conjugate with siRNA has been prepared for more target-specific delivery. The outer layer of HA in the siVEGF/PEI-HA complex was thought to play an essential role in target-specific delivery and better serum stability. This siVEGF/PEI-HA complex can be explored in the treatment of diseases in the tissues with HA receptors, such as liver cancer and kidney cancer.[91,92]

Combined lipid and polymer carriers for a better siRNA delivery

The major components of the delivery systems are cationic lipids and functional peptides and/or a cationic polypeptide like protamine, which can interact with negatively charged siRNA. Surface steric stabilization is introduced by PEGylation to prevent the aggregation of the resulting complex with serum components. Ligands are attached to the distal end of the PEG chain to increase cellular bioavailability. A cationic lipid is necessary for endosome lysis and intracellular release of siRNA. The mechanism of the endosome membrane destabilization is most likely due to the formation of an ion pair complex between the cationic lipid in the nanoparticles and the negatively charged anionic lipids in the endosome membrane, as hypothesized by Xu and Szoka. Also, a short amphipathic peptide, MPG, that is able to form stable nanoparticles with siRNA can enter

the cell independent of the endosomal pathway and can efficiently deliver siRNA in a fully biologically active form into a variety of cell lines and *in vivo*. Another study in March 2009 demonstrated novel lipid-polycation-DNA (LPD) nanoparticles containing DOTAP targeted with polyethylene glycol (PEG) tethered with anisamide (AA) to specifically deliver siRNA to H460 human lung carcinoma cells which express the sigma receptor.[93,94] This specific nanoparticle formulation of siRNA prepared with the new cationic lipid DSGLA targets lung tumor cells and plays both roles of a delivery component and a therapeutic agent. Recently, an immobilized nanoparticle-based platform for an efficient gene knockdown of targeted cells in the circulation has been reported. In this study, P-selectin, an adhesion molecule generated in endothelial and platelet cells, was covalently attached to the surface of DSPEPEG2000-stabilized siRNA-nanoscale liposomes to create targeted nanoparticles for the capture of leukocytes from the bloodstream.[95]

Peptide-mediated siRNA delivery

Many peptide-based siRNA conjugates have been prepared and demonstrated as the better transfection-inducing agents. A small section of proteins called a peptide transduction domain (PTD) has the ability to permeate cell membranes and has been conjugated directly with siRNA for its better cellular entry and reductive release of intact siRNA from the conjugate into the cytoplasm where it specifically targets mRNAs and silences them.[96] Cancer is a complex, genetic disease that is different in every patient; siRNA-induced RNAi approach is the best approach to personalized cancer treatment. The synthetic siRNA can be designed to bind to a single mutation and only that mutation on the genome and it can be easily and rapidly changed while maintaining the delivery system like the PTD-DRBD (peptide transduction domain-double-stranded RNA-binding domain) fusion protein.[8] Well-known examples of these cell-penetrating peptides (CPPs) include TAT transactivator protein from human immunodeficiency virus type-1 (HIV-1), penetratin and transportan. Intrathecal delivery of all these conjugates could efficiently silence the expression of target mRNA, but penetratin-siRNA conjugates cause the induction of innate immunity. Peptide-based siRNA conjugates, composing of carboxylic acid group of the peptide mimetic of IGF1, D-(Cys-Ser-Lys-Cys) was activated and conjugated to an amine group of the 5′-sense strand of siRNA, were formulated for targeting IRS1 (insulin receptor substrate 1) which showed transfection similar to the chol-siRNA conjugate. Peptides have also been utilized for the cell-specific delivery of siRNAs. For example, Shchiffelers *et al*. utilized an integrin-binding RGD peptide coupled to PEGylated polyethyleneimine to direct siRNA uptake to tumor neovalsulature.[97,98] More recently, there was a report that systemically delivered siRNA-peptide conjugates [RVG (rabies virus glycoprotein)-9R-bound antiviral siRNA] can cross the blood-brain barrier (BBB) without inducing inflammatory cytokines or antipeptide antibodies even after repeated administration.[99] A new strategy is based on a short amphipathic peptide, MPG, that is able to form siRNA incorporating nanoparticles with high stability, independent cellular entry and an efficient delivery of biologically active siRNA.

Antibody-mediated siRNA delivery

Antibody-mediated targeted drug delivery systems have high selectiveness toward a target protein on the cell surface and better systemic stability. Song *et al*. developed antibody-protamine fusion proteins as vehicles for receptor-directed delivery of siRNA and demonstrated their therapeutic potential via tumor growth inhibition in an engineered melanoma model. For siRNA therapeutic applications, brain delivery is particularly challenging, because the siRNA must be targeted both across the brain cell membrane and brain capillary endothelial membranes, which form the BBB *in vivo*. PEGylated immunoliposome with surface-immobilized antibodies were targeted to the transferrin receptor expressed in the BBB, and the other antibody was to target insulin receptors expressed in brain cancer.[100] The binding of the transferrin receptor antibody may induce the transcytosis of the immunoliposome across the BBB. The most active molecular Trojan horse for the human BBB is a mAb against the human insulin receptor (HIR), called the HIRMAb. An alternative linker technology, avidin-biotin technology, has been used here to provide a stable linkage between the siRNA and the targeting antibody. The systemic administration of biotinylated siRNAs conjugated to an antibody (streptavidin conjugated) led to the efficient suppression of a reporter gene expression in a rat model bearing an intracranially transplanted brain tumor.[101] The tissue-specific delivery of siRNA could also be achieved by the fusion of antibodies with protamine.[102] One study uses this fusion for an anti-ERbB2-specific single-chain antibody; siRNAs could be targeted to tumor cells expressing the epidermal growth factor receptor ERbB2.

Aptamer-mediated siRNA delivery

Non-protein-based alternatives to antibodies are the selected nucleic-acid-binding species called aptamers that have a potential target efficiency. Generally, aptamers have molecular weights intermediate between small peptides (~1 kDa) and single-chain antibody fragments. (~25 kDa) and can be synthesized chemically for designing target-specific moiety. An aptamer-targeting prostate specific membrane antigen (PSMA), a cellular receptor abundantly expressed in prostate cancer cells, was directly conjugated to siRNA for prostate cell-specific delivery. These anti-PSMA aptamers have been shown to navigate nanoparticles into cells expressing this antigen. Chu *et al*. successfully developed biotinylated siRNA: aptamer via streptavidin: biotin interactions for transfection into PSMA overexpressing cells without using transfection agents.

Another study utilized the intratumoral administration of chimeric double-stranded RNA-aptamer hybrid delivery system, targeting PSMA overexpressing cell receptors, in a mouse tumor xenograft model. After intracellular entry, the hybrid molecule generated siRNAs which were capable to inhibit the expression of polo-like kinase 1 (PLK1) and Bcl-2. Although aptamer-mediated delivery has self-transfecting efficiency, yet their systemic use demands the addition of nuclease stabilizing agents and endosmolytic functionalities.[103,104]

Dendrimer-mediated siRNA delivery

Dendritic molecules are repeatedly branched species that are characterized by structural perfection. Their controllable molecular structure and size, high chemical and structural homogeneity, high ligand and functionality density, wonderful transfection efficiency, etc. pose many advantages over many other delivery systems. Polycationic dendrimers such as poly(amidoamine) (PAMAM) dendrimers have shown a strong binding affinity for RNA molecules and substantial release over a longer period of time.[106] The siRNA-dendrimer complexes (GL3Luc siRNA-G7 complex) showed considerable resistance to RNase degradation and efficient gene silencing, while neither the naked siRNA nor the nonspecific

GL2Luc siRNA-G7 complex showed any gene-silencing effect. Dendrimers can be further evaluated for cell-specific delivery with a multifunctional fan-shaped architecture having one end attached to a cell-specific ligand and the other end for dendrimer promotion and siRNA binding.[106]

Stem cell-based delivery

Although siRNA is a potent and specific therapeutic agent, its delivery can cause immune responses for siRNAs in the vascular or interstitial spaces. Stem cells, found in most of the multicellular organisms, are characterized by the ability of self-renewal through mitotic cell division and differentiating into a diverse range of specialized cell types. A nonimmunogenic siRNA delivery vehicle, human mesenchymal stem cells (hMSCs), has been developed. hMSCs can act as a viable cellular delivery system that has been injected into an organ (the heart) where they integrate into the tissue and form gap junctions with the target cells that mediate the transfer of siRNA from one cell to another.

Light controllable delivery of siRNA

This interesting strategy utilizes the tagging of photolabile cage compounds with siRNA so as to activate siRNA-mediated silencing upon exposure to the UV light trigger at the required time point. Conjugating appropriately selected cage compound at suitable siRNA is the key to successful targeted therapy. Most recently, the commercial availability of NPOM-caged dT phosphoramidite (by Deiters's group) has raised the hope of RNA phosphoramidites that would allow siRNAs to be purchased with intended nucleotides caged.[108,109] Till date, caged siRNAs have only been demonstrated on reporter genes, except for the few photolinker studies on specific targets in zebrafish embryos.[109]

Proprietary delivery platforms

Lots of pharmaceutical companies are coming up with siRNA incorporated in their proprietary delivery platforms as well as licensing their FDA-approved delivery platforms to specialized leading players in the field of RNAi therapeutics. In 2004, Novartis utilized ALZET pumps for P2X3-specific siRNA delivery. This was proven successful in diminishing mechanical hyperalgesia and neuropathic pain by selectively blocking the expression of pain-implicated P2X3 receptor.[110] Later in 2004, an Australian company Benitec, gained exclusive rights to use and sublicense Stanford's so-called Minicircle DNA technology, a nonviral delivery method for Benitec's DNA-directed RNA (ddRNA)-based drugs. Recently in September 2009, Benitec Limited has signed a binding agreement with China-based Biomics Biotechnologies Co. to collaborate on an RNA interference treatment for chronic hepatitis B virus (HBV) infection. Benitec will contribute its proprietary DNA-directed RNAi (ddRNAi) technology, and Biomics of Nantong has developed a full-site siRNA library, drug target screening and identification, siRNA structure modification and drug delivery systems. Another biotechnology company MDRNA is focused on the development and commercialization of therapeutic products based on RNA interference (RNAi). The DiLA2 platform is MDRNA's proprietary platform for creating novel lipids from amino acids. The platform enables MDRNA to tailor the charge, linker and acyl chains in order to optimize the liposome for delivery to the target tissue of interest. In addition, the platform is designed to permit attachment of various peptides to improve a variety of delivery characteristics including nanoparticle formulation, cellular uptake, endosomal release, and cell/tissue targeting. Recently, in January 2009, Abbott Laboratories has licensed Liquidia Technologies' PRINT nanoparticle technology for the delivery of siRNA-based therapeutics, which has the potential to improve the company's cancer portfolio. The two companies will collaborate on the development of the technology, which allows researchers to create nanoparticles of defined size, shape, surface chemistry, and composition. Abbott will be using the technology to develop delivery methods for small interfering RNAs, which has so far proved problematic for researchers.[111] Such strategic acquisitions and collaborations have accelerated the entry of siRNA-based formulations into clinical trials.

Clinical status and regulatory concerns

Right from the first chemical synthesis of siRNA in 2001, through an exhaustive research on animals for applying RNAi in disease treatments and first entry of bevasiranib into clinical trials to now in 2009, there are too many clinical applications for diseases like cancer, viral infections, respiratory diseases, age-related macular degeneration and rare diseases like pachyonychia congenita. Lots of clinical trials are going on and are planned for taking siRNA into the clinic in the treatment of important diseases through various systemic and local routes like skin and mucosal membranes. Table 1 updates the clinical involvement of various pharmaceutical and biotechnological companies and universities leading to direct different siRNA formulations toward FDA approvals. An Australian company, Benitec, is undertaking the first ever human trial of rHIV7-shl-TAR-CCR5RZ formulation for AIDS using lentiviral vectors transduced with hematopoietic stem cells. The rHIV7-shl-TAR-CCR5RZ vector suppresses HIV by expressing three therapeutic nucleic acids that are directed against key steps in HIV replication. Bevasiranib was the first in-class small interfering RNA (siRNA) drug developed by Acuity Pharma and later licensed by Opko Healthcare, designed to silence the genes that produce VEGF, believed to be largely responsible for the vision loss of wet AMD. Although, Opko Health announced in March 2009 to terminate Phase III trial of bevasiranib for treating wet age-related macular degeneration due to disappointing preliminary results, yet indications of activity are encouraging with no systemic or ocular safety issues. Thus the company remains committed to the continued development of siRNA portfolio targeting VEGF, including recently announced VEGFA165b sparing siRNA. Though there is a setback of few failures in clinical stages, the results of a number of other upcoming Phase II clinical trials may help to overpower fears regarding the question on the efficacy of RNAi-based therapeutics and encourage future investments. Until now, there are no safety guidelines addressing siRNA and there is little information available through research engines. Safety assessment of siRNAs is more complex than classical drugs due to the independent responses of delivery vehicle and the double-stranded RNA trigger. However, extensive evaluation of siRNA delivery formulations with respect to biodistribution, subcellular trafficking, and endosomal release at the preclinical stage would be beneficial in avoiding disappointments at the later phases of clinical trials.

Present trend

When the antisense strategy was first introduced, it was recognized that it could represent a specific, systemic gene silencing strategy. Presently, the progressive development of such a strategy has opened up the treatment options of endless variety of human diseases, provided that a particular gene had been identified and characterized for the disease. Understanding of the chemistry of disease-causing specific gene sequence has cleared the path of this strategy as the new "hot" technology. Aggressive research in RNAi and its understanding has enlightened siRNAs as a directing candle to burn disease-causing elements from the cells. RNAi is enjoying its position as the widely used gene-silencing technique in functional genomics. RNAi facilitates the study of genetics in mammalian cells by enabling rapid and efficient generation of knockouts. It is something that is 100 to 1,000 times more potent than previous gene silencing technologies based on RNA. We appreciate the involvement of many companies in RNAi-based therapeutics and would like to take an opportunity here to mention their name to acknowledge their efforts: AiRNA Pharmaceuticals, Allergan, Alnylam Pharmaceuticals, Benitec, BioCandell Therapeutics, Calando Pharmaceuticals, Cenix BioScience, Cequent Pharmaceuticals, Copernicus Therapeutics, Dicerna Pharmaceuticals, Expression Genetics Corporation, General Research Laboratory, Intradigm Corporation, MDRNA, Mirna Therapeutics, MiRagen Therapeutics, Nitto Denko Technical Corporation, Opko Health, Oxford BioMedica, Polyplus Transfection, Quark Pharmaceuticals, Regulus Therapeutics, RXi Therapeutics, Santaris Pharma, Senesco Technologies, Silence Therapeutics, Siranoamics, siRNAsense A/S, Sylentis Pharmaceuticals, Tacere Therapeutics, Targeted Genetics Corporation and Tekmira Pharmaceuticals Corporation. Many companies like Sirna, Alnylam Pharmaceuticals, Ambion, Dharmacon, Qaigen, Allele, and Roche Applied Sciences are hoping to leverage their experience in synthesizing and chemically modifying RNA to produce correct siRNA for RNAi therapeutics. Many others are involved in exploring and promoting innovative structures to reduce off-target effects, improve stability, efficacy, and delivery of RNAi-based therapeutics. Also, many big pharmaceutical companies such as Novartis, Merck, Pfizer, and Roche have started strategic acquisitions and alliances with market leaders. The market for RNAi-based therapeutics is forecast to grow from 2013 onward, as the first products enter the marketplace, to generate sales in excess of US$2.9 billion by 2020.

Table 3: Patent scenario in major US companies involved in siRNA therapeutics

Company	Approximate no. of granted patents	Approximate no. of applications	Trend of patents/applications
Sirna Therapeutics	25	190	1. Conjugates and compositions for cellular delivery of negatively charged molecules 2. Targeted delivery of nucleic acids using various ligands 3. Delivery using lipid nanoparticle-based compositions 4. Synthesis, deprotection, analysis, and purification of RNA and ribozymes, siRNA treatment of diseases 5. Chemically modified short interfering nucleic acid (siNA) 6. Polymearase III-based expression of therapeutic RNAs
Dharmacon	17	141	1. Effective designing of siRNA for gene-specific targeting 2. Functional and hyperfunctional siRNA 3. Modified and stabilized polynucleotides for use in RNA interference 4. Methods and compositions for selecting siRNA of improved functionality
Alnylam	13	26	1. RNAi modulation of ApoB, RSV, MLL-AF4, PIV, etc. 2. Compositions and methods for inhibiting expression of various respiratory virus genes 3. Chemically modified oligonucleotides 4. Method of treating neurodegenerative diseases 5. Glycoconjugates and cationic lipid derivatives of siRNA
Ambion	1	8	1. Methods and compositions for tailing and amplifying RNA and isolating siRNA molecules 2. Methods and kits for sequentially isolating RNA and genomic DNA from cells 3. System and method for electroporating a sample
Silence Therapeutics	1	3	1. Interfering RNA molecules 2. Lipids, lipid complexes, and use thereof
Calando Therapeutics	1	2	1. Inhibitors of ribonucleotide reductase subunit 2 and uses thereof
Nucleonics	0	3	1. Double-stranded RNA structures and constructs, and methods for generating and using the same 2. Conserved Hbv and Hcv sequences useful for gene silencing 3. Methods and constructs for the evaluation of RNAi targets and effector molecules
Roche	6	7	1. Compositions for delivering nucleic acids to cells 2. Compounds for targeting hepatocytes 3. Methods of treating inflammatory diseases
Alcon	1	36	1. RNAi-mediated inhibition of ocular targets 2. RNAi-related inhibition of aquaporin and TNF
MDRNA	0	3	1. Uses of broad-spectrum RNAi therapeutics against influenza 2. Compositions and methods for enhancing the delivery of nucleic acids into cells and for modifying expression of target genes in cells 3. Modification of double-stranded ribonucleic acid molecules

Patent aspects

Many siRNA-based formulations are now being developed with a patent point of view. Greater clarity in the existing patents and license situation is likely to boost investments in RNAi technologies. From a therapeutic standpoint, RNAi produces good knockouts in tissue culture at low concentrations, but there are lots of roadblocks in ways between getting that in tissue culture and therapeutic target. Presently, the main challenge to harness RNAi is its delivery to get enough in the right cells at the right time through an appropriate siRNA design and tagging it with a suitable delivery system. There is little doubt about the potential commercial value of this technology; however, to fully exploit this potential, a carefully planned and executed patent strategy must be employed. Objectives should be to:

(1) explore the requirements for patentability in the United States and other countries and apply these requirements to RNAi technology;
(2) examine key differences between US and international patent systems that may be relevant to patenting RNAi technology in international venues; and
(3) identify the areas of patent coverage that a company will need to acquire if it is to be successful in the RNAi field. In view of the relatively recent emergence of the RNAi field, analysis should be relied heavily on lessons learned from more mature technologies, such as monoclonal antibodies, gene therapy, and antisense technology, in order to identify the strengths and weaknesses of the RNAi patent estate. The thrust in this area can be adjudged from the fact and figures that about 80 patents have been granted in United States in 2009 till date and about 550 patent applications have been published in 2009 and are currently in the examination stage. These patents and applications cover wider aspects of RNAi like gene silencing, preparation techniques of RNAi, etc.; therapeutic aspects of RNAi like use of RNAi for the treatment of allergy disorders, ocular angiogenesis, cancer, cardiovascular problems, CNS disorders, AIDS, etc. and targeting/delivery technologies like nanoparticles both polymer based and lipid based, electroporation-based delivery, carrier-mediated delivery, biodegradable cationic polymer-based delivery, etc. Table 3 gives a glimpse of the US patent scenario of few major companies involved in siRNA technology.[112]

In a nutshell, it can be said that a successful RNAi patent position will require access to patents in the following three areas:

(1) general patents covering relatively broad aspects of RNAi action;
(2) patents for platform technologies for exploiting RNAi; and
(3) focused patents directed to specific diseases, RNAs, and gene targets. While a patent position in one or even two of these areas might put a company into a blocking position, the key to commercial success will be possession of or access to patents in all three areas, as well as a proprietary position in at least one.[112]

Future Directions and Conclusions

The future will see focused and concentrated research in synthetic nanoconstructs composed of polymers, lipids, lipidoids, or conjugates in providing viable a carrier for systemic delivery of siRNA in the clinic. The development of tissue-specific ligands, cell fusogenic agents, will enable target-specific *in vivo* biodistribution and delivery. Many more drug delivery scientists will join global efforts to overcome the most significant barriers to the widespread use of RNAi therapeutics in a clinical setting, and future work will focus on the development of safe and effective delivery materials to ensure the broadest application of RNAi in the clinic. The efforts will also be toward prophylaxis or cure for otherwise untreatable diseases.

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