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In vivo RNAi

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RNAi - a promising tool for target validation studies and therapeutics

The discovery of RNAi and siRNA-mediated gene silencing (1) has led to a spectrum of opportunities for functional genomics, target validation, and the development of siRNA-based therapeutics. RNAi is a naturally occurring process that leads to targeted mRNA degradation, making it a potentially powerful tool for therapeutics and in vivo studies. Introduction of chemically synthesized siRNAs, (21–23 nucleotide (nt), double-stranded siRNAs with 2 nt, 3' overhangs that resemble the products of the RNase III-like enzyme Dicer), can effectively mediate post-transcriptional gene silencing in mammalian cells without inducing interferon responses (2). Synthetic siRNAs, targeted against a variety of genes, have been successfully used in mammalian cells to prevent expression of target mRNA (2, 3). This has raised the possibility of using synthetic siRNA for target-specific gene silencing in vivo, in animal models and in RNAi-based therapeutics (4, 5).

RNAi has been used in vivo for target validation studies in animal disease models (6). In addition, using siRNA-based therapeutics, disease-causing genes could be selectively targeted and gene expression suppressed. There is considerable interest in using such approaches to specifically target genes involved in diseases such as cancer, Alzheimer's disease, inflammatory diseases, and viral diseases. In theory, any disease associated with overexpression of a known gene could be treated using RNAi.

The challenges in achieving optimal in vivo RNAi include effective siRNA delivery and serum stability. Efficient delivery of siRNA is crucial for successful in vivo RNAi and, as siRNA-mediated RNAi has a short duration in vivo, enhancing the longevity of siRNA is also important.

siRNA delivery and silencing in vivo

Many different approaches have been developed for siRNA delivery, and these include methods that deliver viral vectors or synthetic siRNA. Viral vectors have been very effective in cell line-based experiments (7–9). Lentiviral vectors with siRNA expression cassettes have been used for gene silencing in mice (10). The lentiviral vector system can also be used to generate transgenic animals. The system induces endogenous gene silencing allowing functional genomics and target validation studies in vivo. However, there are limitations to the use of viral vectors in animal models, as viral proteins may cause biological effects such as immune responses. To avoid the potential drawbacks of viral vectors, chemically synthesized siRNA has been used to silence target genes in vivo in animal models.

High-pressure, hydrodynamic tail vein injection of nucleic acids, a technique that was originally developed for gene transfer and expression of transgenes in animal models (11), has also been used for siRNA delivery. This method involves rapid injection of large volumes to facilitate siRNA delivery. In studies using this method for siRNA delivery, a reporter plasmid expressing the luciferase gene and a synthetic siRNA duplex targeting luciferase were co-injected into postnatal mice. This resulted in distinct and specific knockdown of luciferase gene expression by more than 80% in several tissues, including liver, kidney, spleen, lung, and pancreas. This selective knockdown could be seen as early as one day after injection (12). In other studies, a modified hydrodynamic transfection method was used to co-inject a luciferase expression plasmid and synthetic siRNA targeted against luciferase directly into the livers of adult mice, and this led to siRNA-mediated inhibition of luciferase expression (13). The researchers also suppressed luciferase expression from a HCV-NS5B-luciferase gene fusion (the firefly luciferase gene fused to the region encoding Hepatitis C virus NS5B viral polymerase), by targeting the NS5B region with synthetic siRNA (13). These results supported the idea that RNAi therapeutics could be used to target human pathogens.

In further experiments demonstrating siRNA-mediated gene knockdown, injection of synthetic siRNA targeting the Fas receptor (Tnfrsf6) gene suppressed Fas expression to almost background levels in mouse hepatocytes. In addition, these mice were protected from death due to fulminant hepatitis induced by agonistic Fas-specific antibody. The siRNA was labeled with Cy5, and 88% of hepatocytes were Cy5 positive after intravenous siRNA injection (14,

15).

Further studies demonstrated that injection of siRNA into the limb veins of mammals resulted in targeted delivery into the limb muscles. Co-injection of siRNA targeting luciferase and luciferase reporter plasmid into the limb veins of mice, rats, and rhesus macaque resulted in effective silencing of the luciferase reporter gene by >95% in the limb muscles of these animals (16).

In an alternative approach, siRNA has been delivered into cells as cationic liposomes complexed with DOTAP (1, 2-dioleoyl, 3-trimethylammonium propane) (18). Intravenous injection of DOTAP complexed with GFP reporter plasmid and GFP-targeted siRNA into adult BALB/C mice led to a significant reduction of GFP mRNA expression. In addition, intraperitoneal injection of TNF-α siRNA formulated with DOTAP into mice offered a significant protective effect from septic shock when subsequently challenged with a lethal dose of lipopolysaccharide (18).

RNAi therapeutics

Recent reports have provided encouraging evidence that synthetic siRNAs could be effective as therapeutics, which would enable rapid progression from gene sequence to production of a therapeutic agent. In breakthrough experiments carried out by Alnylam, siRNA intravenously injected into mice at normal volume and pressure was taken up into several tissues, including liver, jejunum, heart, kidneys, lungs, and fat tissue. Each siRNA was labeled on the sense strand with cholesterol, which facilitated tissue uptake. The siRNA led to knockdown of the endogenous target gene apolipoprotein B, resulting in lowered cholesterol levels (17).

In other research on respiratory syncytial virus and parainfluenza virus in mice, nasal administration of siRNA, both with and without transfection reagents, effectively protected against infection and also resulted in efficient viral inhibition when administered after infection (27).

Recent exciting results reported by Li, B. et al have shown effective reduction of severe acute respiratory syndrome (SARS) symptoms and viral levels after clinically viable intranasal delivery of siRNAs targeting SARS coronavirus (SCV) genes in Rhesus macaque. siRNAs were synthesized by QIAGEN and had previously resulted in highly potent SCV knockdown in cell culture. D5W solution, a clinically appropriate carrier, was used for siRNA delivery. siRNA treatment did not appear to cause toxicity (28). This breakthrough research was listed by Discover magazine as one of their “Top 100 Science Stories of 2005”(30).

The first clinical data for therapeutic RNAi was recently presented by Sirna Therapeutics for its treatment of Age-Related Macular Degeneration (AMD). The siRNA drug under study is targeted to VEGFR-1, a receptor in the pathway that mediates blood vessel growth. Knockdown of VEGFR-1 should reduce the excessive blood vessel growth that detrimentally affects vision in AMD patients. A dose-dependent improvement in vision has been observed in the patients treated so far, vision has not deteriorated in any patient, and the treatment was well tolerated (29).

siRNA stability

Modifications that can stabilize siRNA in serum by conferring nuclease resistance and increasing efficiency and longevity of RNAi without undesirable toxicity are valuable for in vivo RNAi. A variety of chemical modifications that improve the performance of antisense-oligodeoxyribonucleotides have been described (19). Most of these have also been tested for their efficacy in siRNA (20–24). These modifications include:

- Replacement of phosphodiester (PO) with phosphorothioate (PS) linkage
- Introduction of a 2‧Fluoro (2‧F) or a 2‧O-Methyl modification (2‧O-Me) on the 2‧position of the ribose sugar
- Introduction of modified bases that enhance the A剖 base pairing, such as 5-Bromo-Uridine (BU), 5-Iodo-Uridine (IU) , or 2,6-Diamino Purine (2,6-DAP)

siRNAs with these modifications have been evaluated in cell culture for functionality and nuclease resistance. In particular, siRNA duplexes with a 2’-Fluoro substitution (such as 2'-Fluoro-Uridine (2'-FU) or 2'-Fluoro-Cytidine (2'-FC)) were reported to be functionally active with enhanced stability in HeLa cell extracts, and were considered to be modifications that could significantly prolong RNAi-mediated gene silencing in vivo (24). This possibility was recently tested in animal models. The results suggested that 2’-F modified siRNA was functional in vivo and showed

increased stability in human plasma relative to unmodified 2'-OH siRNA. However, these modifications did not offer any benefit in enhancing the magnitude or duration of silencing, either in cell culture or in vivo in mice, compared to unmodified siRNA (25).

In recent in vivo studies in mice, siRNA with partial phosphorothioate backbone and 2'-O-Methyl modifications on the sense and antisense strands had enhanced resistance to nuclease digestion in serum and tissue homogenates. The same study showed that a cholesterol modification at the 3' end of the sense strand significantly improved in vivo pharmacokinetic properties (17).

Although, in principle, chemical modification that allows an siRNA to be both functional and stable against nuclease attack should be beneficial, often these modifications show little advantage for silencing in vivo. The transient nature of synthetic siRNA-mediated RNAi in proliferating cells may also be due to the dilution of siRNA in these cells as they divide (26). Future work on alternative delivery approaches or disease models may reveal methods to optimize the benefits of chemically modified siRNA.

The future of in vivo RNAi

Future RNAi therapy will probably be most successful for diseases caused by a single gene or a small number of genes or for use as a complementary therapy in treatment of complex multigenic disorders. Optimizing siRNA delivery is one of the biggest challenges for successful in vivo drug target validation in animal models and for RNAi therapies. Successful siRNA therapeutics may be more easily achieved when siRNA is delivered directly to specific organs, for example injection of siRNA into the eye to treat AMD. This approach has the advantage that the risk of side effects elsewhere in the body would be minimized.

In addition, increased understanding of the RNAi mechanism, turnover of siRISC (siRNA-dependent RNA-induced silencing complex), and kinetic parameters will also help to improve siRNA formulations.

Many companies are involved in the development of RNAi therapies, including Sirna therapeutics, who specialize in AMD and Hepatitis C; Alnylam pharmaceuticals, specializing in AMD and Parkinson's disease; and Intradigm, specializing in cancer. With encouraging results from animal studies and Phase I clinical trials for Sirna's hepatitis C treatment planned for late 2006, RNAi techniques are showing the potential to revolutionize therapeutics as they have for in vitro functional genomics.

siRNA from QIAGEN

Economical, high-purity siRNA from QIAGEN is compatible with in vivo animal delivery systems. siRNA is synthesized using proprietary synthesis and purification processes, yielding siRNA that is >90% pure. The high-throughput synthesis and purification processes enable fast turnaround times and high yields, and each siRNA duplex undergoes stringent quality control analysis. Large-scale synthesis of high-quality siRNA is available for in vivo animal studies at a range of siRNA scales from 10 mg-10 g and with a variety of modification options.

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