TECH NOTE

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In vivo RNAi: Biodistribution, delivery, and applications

Introduction

RNA interference (RNAi) is an extremely valuable tool for the study of gene function, gene identification, and biological pathways. RNAi is an evolutionarily conserved anti-viral response that is triggered by an externally introduced double stranded RNA (dsRNA). The dsRNA is cleaved by the RNase-III enzyme, Dicer, into small interfering RNAs (siRNA) that are approximately 21-23 nt. The siRNA then loads into an RNA-Induced Silencing Complex (RISC), which facilitates the separation of the two siRNA strands and cleavage of the target mRNA.

Synthetic siRNAs are used to suppress gene expression in mammalian cells without inducing an interferon response. siRNAs can be specifically designed to target any gene and can silence target mRNA expression often greater than 90%. The success of siRNA-mediated gene silencing in cell culture has led to the next application of RNAi, which is the use of synthetic siRNA for target-specific gene silencing in animals.

In vivo RNAi has been used for target validation studies in animal disease models[1], and also has the potential to be used as siRNA therapeutics, where disease-causing genes could be selectively targeted and the gene expression suppressed. Diseases such as Alzheimers, cancer, age-related macular degeneration, retinopathy, asthma, chronic obstructive pulmonary disease, SARS, and rheumatoid arthritis would greatly benefit from siRNA therapeutics as these diseases are due to aberrant gene expression and the elimination of this gene expression could alleviate the disease.

Biodistribution

There are two main strategies for *in vivo* siRNA delivery, systemic or localized, which result in very different biodistribution profiles. When delivered systemically, the typical clearance time for siRNA without any conjugates or modifications is less than ten minutes in the body. Due to this short residence time, the choice of delivery method and the resulting biodistribution is very important.

Systemic delivery, as the name implies, is an injection into the systemic circulation system, which provides widespread distribution of siRNA throughout the animal. Systemic delivery includes tail vein injection and intraperitoneal (abdominal cavity) injection[2,3,4,5].

Tail vein injections involve a rapid injection of either a large volume (10% volume/weight, or high pressure) or a lower volume (1% volume/weight, or low pressure) of siRNA into the tail vein.

Localized delivery is injection of siRNA directly into the target areas, thus providing more limited biodistribution. The advantage of local delivery is the ability to better direct the siRNA to the cells or tissues expressing the target gene of interest. Successful applications of localized delivery include intranasal[6], intrathecal[7,8], intratesticular[9], intraliver[10,11], intramuscular[12,13], intraretinal[14,15], and intratumoral[16,17].

The physical stability of the siRNA is another consideration for good biodistribution. Unmodified siRNAs have a half-life of less than 10 minutes in 100% human serum due to their degradation by nucleases (Figure 1). Thus, nuclease resistance of siRNA is important for efficient target knockdown. This is especially true when siRNA is systemically delivered, as there is an increased transport time to the target cells. Modified siRNAs are available that improve the nuclease resistance, such as Dharmacon™ siSTABLE™ siRNA, which has stability for greater than five days in 100% human serum.

Another consideration for *in vivo* RNAi is the siRNA dose administered. In general, a larger siRNA dose is used for systemic injection compared to localized injection. However, there are currently no specific recommendations. Current publications report systemic doses that range from 0.01-50 mg/kg/day, and localized doses that range from 0.01-4 mg/kg/day.

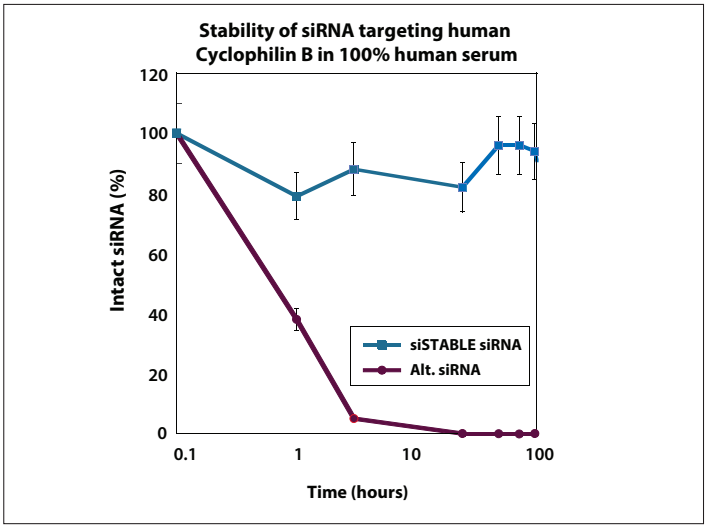

Figure 1. Stability of unmodified and siSTABLE v2 modified siRNA in 100% human serum. Unmodified siRNA degrades almost immediately while siSTABLE siRNA maintains integrity for up to 5 days.

Delivery reagents

Delivery reagents that facilitate efficient siRNA entry into cells include siRNA conjugates and complexes. Conjugates such as cholesterol and peptides can be covalently linked to the chemically stabilized siRNA molecule to aid in its uptake into tissues[3]. Several delivery reagents that complex siRNA to facilitate cellular uptake are cationic liposomes, dendrimers, and atelocollagen [2,9]. Cationic liposomes have been relatively successful in cell culture, although they require lengthy optimization of transfection conditions. Limitations of cationic lipids include the lack of ability to transfect a vast number of cell types and undesirable levels of toxicity. For *in vivo* applications, cationic liposomes have been shown to be less effective and more toxic. Compared to cationic lipids, polymer-based dendrimers display greater transfection efficiency and less toxicity in many cell types, thus they may have a greater potential for *in vivo* application. Finally, atelocollagen, a naturally occurring protein that is low in immunogenicity, is used clinically for a wide range of purposes. Atelocollagen has been shown to allow increased cellular uptake, nuclease resistance and prolonged release of genes and oligonucleotides. Atelocollagen displays low-toxicity and low-immunogenicity when transplanted *in vivo*.

Additionally, electroporation has been used for localized delivery to skin and muscle as it is effective with nearly all cell and species type and may be performed with intact tissue[12,13,15]. However, there are several disadvantages to electroporation including cell damage, rupture, and cell death.

Detection methods

The method of knockdown detection will depend on whether the siRNA molecule itself or the activity of the siRNA is being measured. For detection of the siRNA molecule and to measure its uptake, a label such as fluorescence or radioactivity can be attached. The fluorescent-labeled siRNA molecule may be easy to synthesize and use, but it is possible that the label will change the siRNA pharmacokinetics. In contrast, radioactivity uptake provides good biodistribution data, but it has regulatory requirements and it poses technical and handling challenges. Non-fluorescent labels that can be detected immunologically or enzymatically include biotin, digoxygenin, and BrDU (bromodeoxyuridine).

Alternatively, a transgenic mouse expressing EGFP can be used to visualize the effect of EGFP siRNA. For the detection of the siRNA activity, either the phenotype of the knockdown or target mRNA/protein levels can be analyzed. Although a phenotypic assay is usually quite simple, the resulting phenotype may be caused by multiple factors and it is not a direct assessment of the mRNA knockdown. Therefore, the detection of mRNA and protein levels is the most reliable method for determining siRNA functionality and biodistribution. However, the isolation of mRNA and protein *in vivo* is much more difficult than in cell culture.

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