



Optimization of Macromolecular Design for siRNA Delivery

Jeff Sparks, Gregory Slobodkin, Majed Matar, Jason Fewell, Jennifer Rice, Elaine Brunhoeber, Casey Pence, Diane McClure, Khurshheed Anwer
Expression Genetics, Inc., Huntsville, AL



ABSTRACT

One of the most prominent differences between the delivery of plasmid DNA and the delivery of siRNA is the size of the two payloads, and is a primary consideration driving the design of non-viral vectors for delivery of siRNA. The small size of siRNA results in a unique interaction with the delivery system. We hypothesize that the stability of formulated siRNA complexes becomes an important parameter in vivo, in part due to the presence of competitive binders, both for the siRNA and for the delivery system. Our approach was to improve our understanding of the structure/function relationship between siRNA and various delivery systems. As a starting point, delivery systems which worked well in plasmid DNA applications in the past were evaluated for siRNA delivery in comparison to new synthetic variants in which general core properties of the delivery systems were dialed. It was observed that the molecular configuration and complexity of the delivery system significantly influenced siRNA stability and knockdown efficiency in cell culture and tumor explants. Interestingly, those systems which showed high levels of knockdown with siRNA showed only moderate to little expression when formulated with plasmid DNA. These systems which showed good knockdown efficiency (~50%) in cell culture were then tested in vivo by systemic delivery targeting VEGF transcripts. The most promising candidates screened previously showed significant knockdown in the lung compared to non-coding siRNAs. These results support our idea that new structural paradigms must be developed for siRNA delivery. We have examined the effects of novel technologies incorporated into delivery systems, which were designed to promote specific interaction with smaller siRNAs, thereby providing stability and protection of the siRNA and to allow cellular uptake in vivo. We sought to understand this change in delivery efficiency as plasmid DNA was replaced with siRNA. A series of experiments were designed to rapidly and economically screen new delivery systems which served to simulate in vivo conditions and which allowed tracking both delivery systems and siRNA using fluorescent tags. We were able to show that distinct molecular features in the delivery systems influence their ability to perform better with siRNA compared to plasmid DNA by favorably affecting the nature of the complex following delivery. A detailed structure/function analysis to better understand the relationship between the molecular configuration of the delivery systems and the physico-chemical properties and knockdown efficiency of siRNA complexes will be presented in context with their in vitro and in vivo application.

INTRODUCTION

The ability to silence target genes at the mRNA level using native intracellular machinery has allowed RNAi to develop into a new class of therapeutics. In the past, several siRNA sequences have been targeted through RNA hybridization and had been explored with antisense technology. Though the possibilities for such an approach were intriguing, general utility of antisense was never fully realized. The potential to use a naturally occurring pathway to alter expression levels potentially makes RNAi more efficient and therapeutically promising. However, as is the case with the majority of biological therapies, delivery of the drug to target tissues presents a formidable challenge.

Over the past two decades, many different types of non-viral systems have been developed as a means to deliver plasmid DNA into cultured cells in vitro and into target tissue in vivo. The goal of this approach was to achieve gene expression by transferring the DNA into the nucleus of the cell, and macromolecules such as cationic lipids and functionalized polymers proved successful in many instances. With the advent of the RNAi era in the late 1990s came the need to deliver a different type of nucleic acid, short (~21 bp) segments of double stranded RNA (siRNA), into the cytoplasm of the cell.¹⁻⁴ However, many of the best plasmid DNA delivery systems were largely unsatisfactory when used to deliver siRNA. Consequently, novel delivery systems need to be developed that are rationally designed based on a fundamental understanding of siRNA delivery requirements. Properties unique to siRNA therapies include sensitivity to extracellular RNAses, activation of the immune system through recognition by Toll-like receptors, and non-specific gene knockdown through off-target effects.⁵ However, there are several advantages to working with siRNA, primarily due to the fact that they are made synthetically. The backbone may be modified chemically to impart stability, ligands and modified end groups may be added during solid phase synthesis, and due to their short lengths, several unique sequences may be easily generated to target a single gene. In addition, due to their small size compared to plasmid DNA, they may be considered more like small molecules in their behavior and less like large plasmids in traditional gene therapy. These properties are used to guide the rational design of non viral delivery systems.

Here we present data detailing the development process of several proprietary delivery systems designed expressly for siRNA. A comparison of their performance with systems designed for plasmid DNA is presented, together with a series of experiments which highlight fundamental differences between their interactions with plasmid DNA and siRNA. Initial in vivo data for the best system determined by simulated in vivo conditions in vitro is also described.

METHODS

All plasmid constructs were purified using the Qiagen EndoFree plasmid giga kit (Qiagen). All siRNAs were obtained from Dharmacon. Quantification of mVEGF protein levels was performed by ELISA using commercially available ELISA kits (R&D Systems). GAPDH protein levels were measured using a GAPDH quantification kit from Ambion. RT-PCR was performed using an Applied Biosystems ABI 7300 system. All procedures involving animals were undertaken in compliance with the Federal and local principles and procedures and overseen by the Institutional Animal Care and Use Committee (IACUC).

RESULTS

Plasmid-Specific Delivery System Used to Deliver Plasmid DNA vs. siRNA

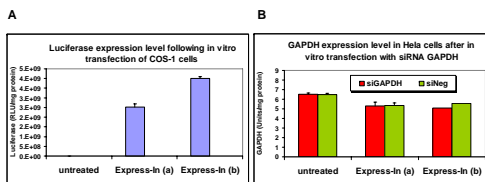


Figure 1.

Two proprietary EGEN polymeric formulations, which are sold commercially as Express-In™ (Open Biosystems) for general plasmid DNA transfection, were examined for their ability to cause GAPDH knockdown in vitro using siRNA. (A) Express-in (a) and Express-in (b) were used to deliver luciferase-encoded plasmid DNA to COS-1 cells. (B) The same formulations were used to deliver siRNA targeting GAPDH to HeLa cells. The values expressed are mean ± SD and are normalized to total protein levels per well.

siRNA-Specific Delivery Systems Used to Deliver Plasmid DNA vs. siRNA

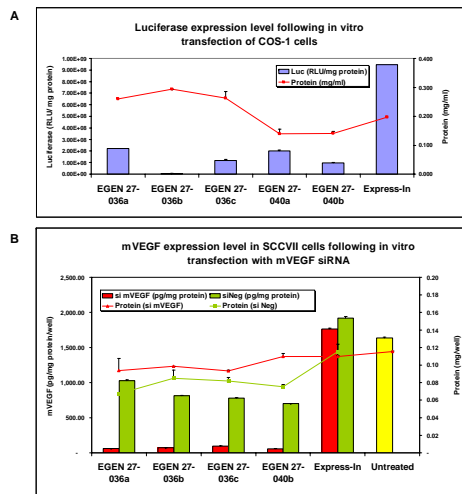


Figure 2.

Activity of novel EGEN delivery systems specifically designed for siRNA delivery. (A) EGEN delivery systems, including those specific for plasmid DNA (Express-In™) and those specific for siRNA were formulated with plasmid DNA encoding luciferase and administered to COS-1 cells. (B) The same systems were formulated with mVEGF siRNA and administered to SCCVII (equamous cell carcinoma) cells. The values expressed are mean ± SD and are normalized to total protein levels per well.

Performance of siRNA-Specific Delivery Systems in Simulated In Vivo Conditions

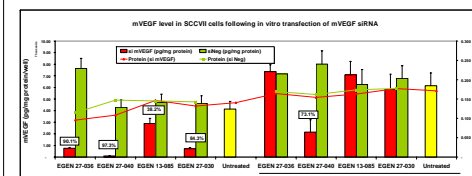


Figure 3.

In vitro knockdown of mVEGF with EGEN siRNA-specific delivery systems under conventional transfection conditions (left) and under simulated in vivo conditions (right). The values expressed are mean ± SD and are normalized to total protein levels per well.

mVEGF Levels Following IV Injection of mVEGF siRNA Formulated With EGEN 27-040b

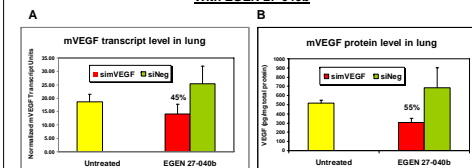


Figure 4.

IV injection of mVEGF formulated with EGEN 27-040b into the tail vein of ICR mice (n = 5 per group). Dose: 300 µl of a 0.3 µg/ml solution at 4.5 mg/kg per animal. Once daily doses were administered for 3 days. (A) mVEGF transcript was measured 24 h after the first injection; transcript units were normalized to ribosomal RNA. (B) mVEGF protein was measured 24 h after the third injection; protein levels were normalized to total protein. All knockdown levels are reported relative to noncoding siRNA controls.

- Plasmid-specific proprietary delivery system, Express-In™, was shown to result in high levels of luciferase expression in vitro when formulated with plasmid DNA encoding luciferase, but low levels of VEGF knockdown in vitro when formulated with siRNA targeting GAPDH (Figure 1).
- New siRNA-specific delivery systems were synthesized and were found to significantly inhibit VEGF transcript and protein in vivo when formulated with siRNA targeting mVEGF, but resulted in low levels of luciferase expression in vitro when formulated with plasmid DNA encoding luciferase (Figure 2).
- Promising candidates from a pool of synthetic variants were screened for in vitro activity under traditional transfection conditions, and were examined under simulated in vivo conditions. One delivery system, EGEN 27-040, was found to retain much of its activity under the stringent simulated in vivo conditions (Figure 3).
- IV administration of mVEGF siRNA formulated with EGEN 27-040b resulted in significant sequence-specific knockdown of both mVEGF transcript and mVEGF protein levels in the lung and may indicate a use in therapeutic applications (Figure 4).
- Unique structural attributes are required for siRNA vs. plasmid DNA for in vivo delivery.

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