

# Gene silencing in the therapy of influenza and other respiratory diseases: Targeting to RNase P by use of External Guide Sequences (EGS)

David H Dreyfus<sup>1,2</sup>  
S Mark Tompkins<sup>3</sup>  
Ramsay Fuleihan<sup>1</sup>  
Lucy Y Ghoda<sup>2,4</sup>

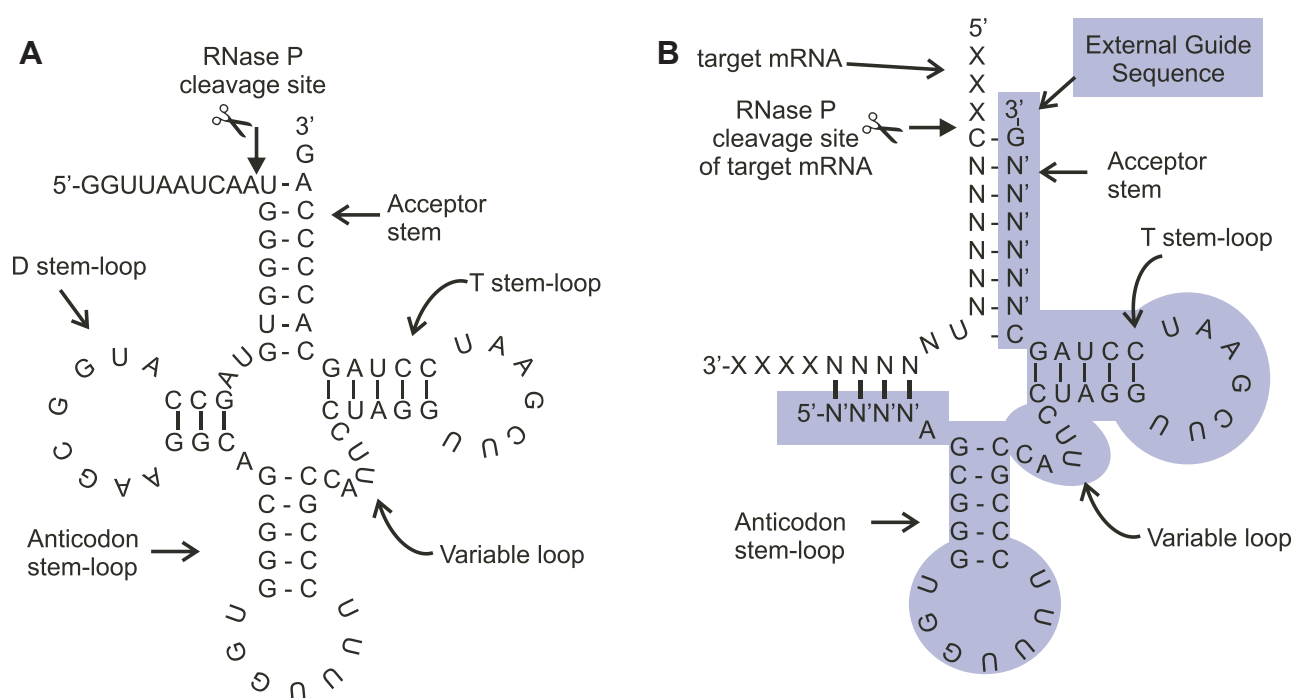
<sup>1</sup>Department of Pediatrics, Yale University School of Medicine, New Haven, CT, USA; <sup>2</sup>Keren Pharmaceuticals, New Haven, CT, USA; <sup>3</sup>Department of Infectious Diseases, University of Georgia College of Veterinary Medicine, Athens, GA, USA; <sup>4</sup>The Webb-Waring Institute and the Department of Medicine, University of Colorado Health Sciences Center, Denver, CO

**Abstract:** Respiratory diseases provide an attractive target for gene silencing using small nucleic acids since the respiratory epithelium can be reached by inhalation therapy. Natural surfactant appears to facilitate the uptake and distribution of these types of molecules making aerosolized nucleic acids a possible new class of therapeutics. This article will review the rationale for the use of External Guide Sequence (EGS) in targeting specific mRNA molecules for RNase P-mediated intracellular destruction. Specific destruction of target mRNA results in gene-specific silencing similar to that instigated by siRNA via the RISC complex. The application of EGS molecules specific for influenza genes are discussed as well as the potential for synergy with siRNA. Furthermore, EGS could be adapted to target other respiratory diseases of viral etiology as well as conditions such as asthma.

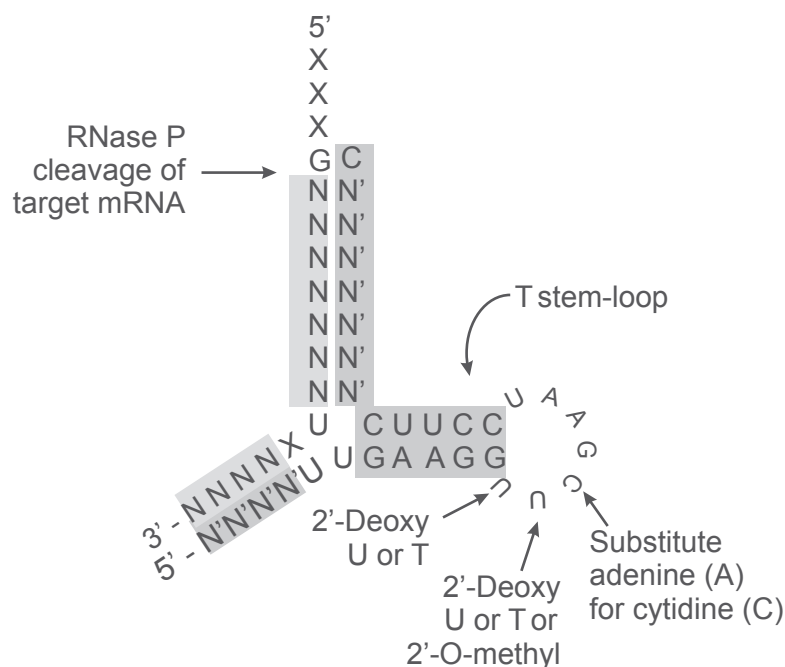
This article will review the use of External Guide Sequence (EGS) directed gene expression silencing which promotes messenger RNA (mRNA) cleavage by RNase P, and the potential applications of this technology to treatment and prophylaxis of influenza and other respiratory diseases such as post viral exacerbations of asthma (Dreyfus et al 2004; Nyce 1997; Nyce and Metzger 1997; Popescu 2005; Trian et al 2006; Ulanova et al 2006). Gene silencing with RNase P is based upon the discovery that the ubiquitous RNA enzyme RNase P, required for processing of precursor transfer RNA (pre-tRNA) to the mature transfer RNA (tRNA) can also be programmed to degrade mRNA (Baer et al 1988; Gopalan et al 2002; Guerrier-Takada et al 1988; Guerrier-Takada et al 1989; Guerrier-Takada and Altman 2000; Raj and Liu 2003). Since RNase P recognizes the structure of pre-tRNA and certain conserved sequence elements of that structure (Figure 1A), a single stranded RNA termed EGS, can be designed to bind non-covalently to target mRNA resulting in a bimolecular structure that resembles pre-tRNA and is recognized and cleaved by RNase P (Figure 1B). EGSs, as a therapeutic agent, fall into the category of oligonucleotides – respiratory diseases as a whole may be particularly amenable to gene silencing using small nucleic acids since the respiratory epithelium is accessible by inhalation delivery and appears to spontaneously take up these types of molecules (Finotto, Buerke et al 2001; Finotto, De Sanctis et al 2001; Massaro et al 2004; Moschos et al 2007; Nyce 1997; Thomas et al 2007).

EGS, a short single-stranded RNA, binds by Watson-Crick base-pairing to target mRNA and directs it to RNase P (Guerrier-Takada and Altman 2000). The bimolecular structure of EGS and mRNA forms a substrate for site specific cleavage of the mRNA moiety and inactivation of the mRNA target (Figure 2). After cleavage of the target, the EGS is released and can bind new target, thereby facilitating additional cycles of target cleavage. The rate of scissile bond cleavage is limited primarily by the concentrations of EGS and

Correspondence: Lucy Y Ghoda  
The Webb-Waring Institute, UCDHSC,  
4200 East Ninth Ave, Campus Box C321,  
Denver, CO 80262, USA  
Tel +1 303 315 7961  
Email [lucy.ghoda@uchsc.edu](mailto:lucy.ghoda@uchsc.edu)



**Figure 1 (A)** The structure of pre-tRNA (Gln): The structure of precursor transfer RNA (pre-tRNA), a typical substrate for RNase P. RNase P is an abundant RNA enzyme that processes a precursor tRNA transcript through cleavage of a 5' leader sequence from the transcript. Following processing, the tRNA can accept an amino acid and function in protein synthesis. **(B)** A synthetic external guide sequence (EGS, highlighted) derived from the structure of pre-tRNA (Gln, Figure 1A) bound to target mRNA forms a pre-tRNA (Gln)-like structure resulting in cleavage of a non-natural substrate mRNA (eg, target). RNase P recognizes the structure as precursor RNA and cleaves the mRNA (depicted as scissors). Thus an EGS can be designed that binds to a target mRNA through altered stem sequences maintaining a conserved stem and loop structure resembling a tRNA precursor. The EGS mRNA hybrid is then recognized as a substrate for RNase P.



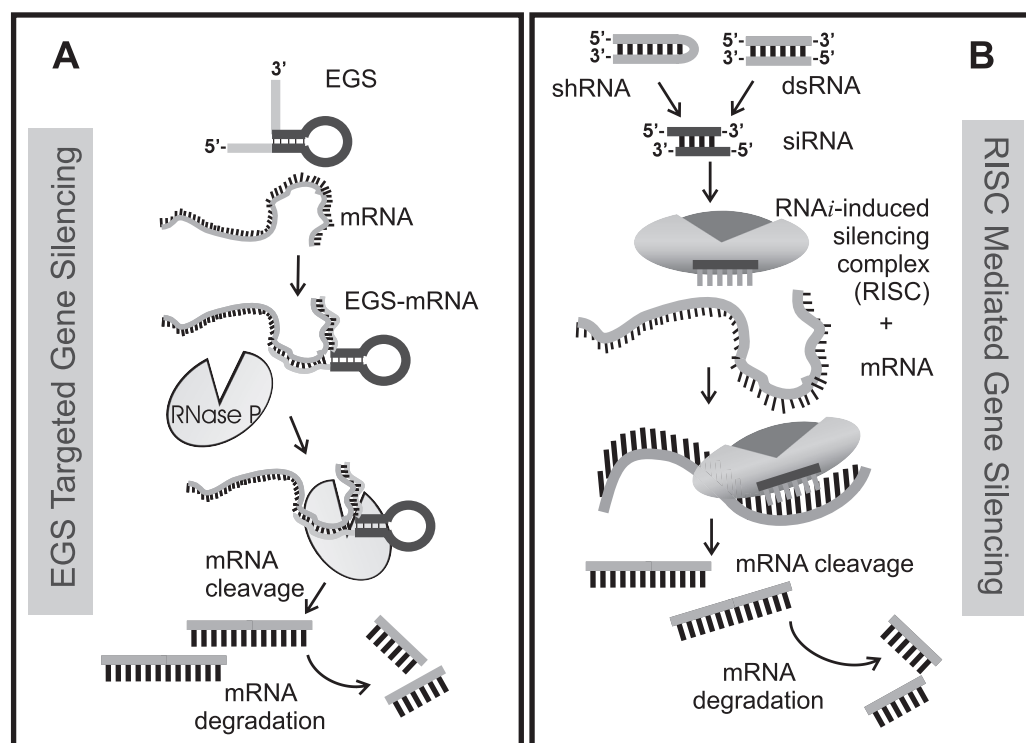
**Figure 2** EGS can be minimized to approximately 32 nucleotides of single stranded nuclease resistant RNA by incorporation of modified bases conserving the T stem loop necessary for recognition by RNase P. The yellow highlighted nucleotides correspond to regions in the target sequences homologous to the EGS. The pink highlighted regions are 2'-O-methyl modified nuclease resistant nucleotide moieties. The minimized EGS contains only the T stem-loop structure. Other sequences such as the variable and anticodon loops of tRNA are not required for cleavage by RNase P but may affect binding affinity.

mRNA species complementary to the EGS in the nucleus and the binding affinity (Km) of the bimolecular structure to RNase P, which for the native pre-tRNA is a relatively low 9 nM (Ziehler et al 2000). RNase P concentrations are high because it is utilized for generation of tRNA in all cell types with a nucleus and active protein synthesis, thus this molecule is not rate-limiting in the reaction (Doersen et al 1985; Koski et al 1976; Robertson et al 1972). In HeLa cells, it is estimated there are 20,000 copies of RNase P per cell (Bartkiewicz et al 1989).

EGS can be expressed from viral or other expression systems in different cell types and directed at different targets (Dreyfus et al 2004; Kovrigina et al 2005; Zhang and Altman 2004; Kovrigina et al 2003; Guerrier-Takada and Altman 2000; Plehn-Dujowich and Altman 1998; Li et al 2006; Barnor et al 2004; Zhu et al 2004; Kitano et al 2000; Endo et al 2001; Kraus et al 2002; Hnatyszyn et al 2001). Alternatively, EGS can be transiently expressed following high copy number transfection or electroporation of plasmid containing an RNA Pol III promoter driving the EGS sequence (Dreyfus et al 2004; Rangarajan et al 2004). For therapeutic applications in humans such as for asthma or influenza, EGS can be chemically synthesized as nuclease resistant small

molecules (Ma et al 1998, 2000; Zhu et al 2004), avoiding the need for vector and promoter sequences (Figure 3). Transient transfection of these nuclease resistant molecules circumvents the risk of somatic gene mutations which could lead to malignant transformation of recipient cells when viruses or virally derived DNA sequences are used.

In a research setting, using EGS expressed at high copy number *in vivo* from vectors is more convenient and cost effective for screening large numbers of constructs than using stably transfected cell lines or transfection of chemically synthesized EGS (Dreyfus et al 2004). We described a relatively simple screening assay in which EGS and reporter genes are transiently electroporated into a well characterized and publicly available B-lymphoblastoid cell line that expresses a functional IL-4 receptor to demonstrate the ability of EGS to silence IL-4 signaling using the human IL-4 promoter fused to the luciferase gene. We have also used a Jurkat T lymphoblastoid cell line expressing both functional IL-4 and adenosine receptors important mediators of asthma and other respiratory diseases (Georas et al 1998), to confirm these results. In this type of *in vitro* cellular assay, EGS potency and specificity are assessed by silencing of a luciferase reporter



**Figure 3** Comparison of (A) EGS directed targeting of mRNA to RNase P with (B) small double-stranded RNA (dsRNA) or regulatory short hairpin RNA (shRNA) directed targeting of mRNA to RISC. Both pathways require a nucleotide guide sequence – for RNase P, EGS is the guide while for RISC, the antisense strand of a double-stranded small interfering RNA (siRNA) is incorporated into the RISC complex. siRNAs can be directly introduced into the cell or are generated by the enzyme, Dicer from short hairpin RNA generated from a DNA template or from dsRNA. In this view, the EGS that binds to target mRNA and forms a substrate recognized by RNase P is directly analogous by function to the antisense strand of the 21–23 nucleotide siRNA that is incorporated into RISC and guides Slicer mediated mRNA cleavage. Both RNase P and the argonaute protein Slicer introduce a single cleavage site in the target – the cleaved mRNA is recognized as abnormal and further degraded by cellular nucleases.

gene. Moreover, the assay can be scaled up and automated for large numbers of candidate EGS.

The relevance of IL-4/13 receptor as a target for EGS or other gene silencing has been reviewed previously (Dreyfus et al 2004). The effects of active EGS that are RNase P dependent can be distinguished from non-specific effects of the EGS on reporter expression since they are decreased by mutation of the EGS T loop required for cleavage by RNase P. Some interesting conclusions are evident regarding potency and specificity of the anti-IL4 receptor EGS in this type of assay (Dreyfus and Fuleihan, unpublished observations). Two EGS molecules designed to target two distinct regions in the IL-4/13 common receptor mRNA each independently exhibited RNase P dependent inactivation of IL-4 signaling. Of the two EGSs, one which targets a site near the start codon of the IL-4/13 common receptor mRNA is more potent than another EGS targeting a sequence located more distal to the start codon even though both target sequences are similarly accessible based on digestion by nuclease T1 *in vitro*. Also, an EGS targeting the murine homologue of the human IL-4/13 receptor chain was still able to cause RNase P dependent inactivation of the human target despite the presence of mismatches in 2 of 11 nucleotides. However, these mismatches resulted in a greater than 50% decrease in target site inactivation when tested in tissue culture.

Thus, RNase P dependent EGS targeting is dependent on multiple factors related to target site accessibility and conformation *in vivo* – factors that are not entirely predictable using sequence based algorithms or *in vitro* methods. Targets can be cleaved *in vivo* even if significant mismatches are present between EGS and target although this cleavage is less efficient than correctly paired EGS. Methods such as DNA microarray analysis could be used to look for evidence of off target/non-specific effects of EGS. Once an optimal candidate EGS or multiple EGS are identified using an unbiased automated process capable of screening large numbers of randomized or otherwise modified EGS, these optimized EGS would then be further characterized *in vitro* and *in vivo* in human primary cells and animal models prior to human clinical trials. In the absence of a readily available reporter gene for monitoring EGS activity *in vivo*, a reporter gene could be constructed which expresses the target gene mRNA fused upstream of the luciferase mRNA so that cleavage of the target mRNA would proportionately reduce luciferase expression.

For *in vivo* applications, EGS delivery methods must be optimized to permit sufficient concentrations of EGS to enter the respiratory tract and gain access to cells of the respiratory epithelium as nuclease resistant small molecules with

minimal non-specific or pro-inflammatory properties. To accomplish this, an inhaler could be optimized for delivery of EGS in a transfection reagent composed of lipids. The concentration of EGS in the cell nuclei will be the limiting factor in the rate of EGS-directed target cleavage. Since EGS molecules are RNA oligonucleotides, many of the issues encountered in delivering siRNA are applicable to EGS. In the case of siRNA, published data suggest a therapeutic effect against influenza even with the administration of unmodified RNA duplex by hydrodynamic tail vein injection in mice (Tompkins et al 2004; Larson et al 2007; Herweijer and Wolff 2007; Bradley et al 2005).

RNAi is an evolutionarily conserved expression silencing pathway which also plays a role in antiviral defense, particularly against those viruses with an RNA genome that replicate in the cytoplasm. However, RNA viruses such as influenza and vaccinia have developed means of undermining the RNAi pathway (Li and Ding 2005; Schott et al 2005; Schutz and Sarnow 2006). Viral proteins often target the enzymes in RISC, thereby attenuating the RNAi pathway rather than directly targeting the regulatory RNA mediator molecules. While the role of RNase P in antiviral defense has not specifically been explored, it is interesting to note that a putative RNase P RNA gene has been identified in camelpox and vaccinia viruses, members of the orthopoxviruses (Yang et al 2005). These RNase P molecules were found to be enzymatically inactive and a role for these RNA enzymes in the viral life cycle has yet to be established.

We suggest that synergy may be possible between gene silencing with EGS/RNase P and gene silencing with siRNA/RNAi. As shown in Figure 3, gene silencing with EGS and RNase P and gene silencing with siRNA and the RNA-induced silencing complex (RISC) share similar characteristics. In both cases a small RNA sequence with complementarity to the target serves as a guide to cleavage of the mRNA by an endogenous RNase. Both schemes result in the cleavage of many targets per guide sequence. In the RNase P mediated gene silencing pathway, the small single stranded RNA is termed an EGS, while with RISC, the RNA is termed siRNA. In contrast, antisense technology utilizes modified DNA oligonucleotides which act pleiotropically at the level of transcription, splicing, protein synthesis and can also activate RNase H resulting in destruction of the complementary mRNA (Monia et al 1996; Monia et al 1993; Lima et al 1992; Chan et al 2006).

What then are the relative advantages and disadvantages of the RNase P pathway compared to RNAi? Advantages of gene silencing with EGS/RNase P may include more rapid

onset of action than siRNA/RNAi (Zhang and Altman 2004). Experiments using EGS and shRNA expressed from identical vectors against an identical target suggest that the onset of action of EGS can be detected within 24 hours while under identical conditions, shRNA effects were evident only after 48 hours. If this 24 hour difference in onset of action is a general phenomenon that distinguishes the two, it could be highly significant for therapeutic applications. For example, in the treatment of respiratory viral infections such as influenza, a difference in onset of action of 24 hours could result in significantly different outcomes in viral titer as well as severity of symptoms and complications. In addition, where a gene silencing strategy is used for prophylactic purposes following exposure to virus, a 24 hour difference in onset of action could be highly significant since viral replication peaks within 48 hours of exposure.

Another potential advantage is that EGS is essentially single-stranded. Thus, Toll receptor 3 recognizing short, double-stranded RNA would not be expected to be activated (Kariko, Bhuyan et al 2004; Kariko, Ni et al 2004; Akira 2006; Matsukura et al 2006).

Another important point of comparison between EGS and siRNA is potency and specificity which are related, but not identical issues. A less potent drug can have a similar efficacy to a more potent drug if a higher concentration of the less potent compound is used. However, where there are significant off-target effects, increasing dosage will almost certainly lead to more non-specific side-effects such as inflammatory responses or the triggering of pro-apoptotic proteins such as p21 in the case of siRNA (Bridge et al 2003; Jackson et al 2003; Jackson and Linsley 2004; Scacheri et al 2004). At the current time, very little is known about the specificity of either siRNA or EGS under in vivo conditions and particularly in the inflammatory setting predicted to be elicited by a viral infection such as influenza, or a chronic inflammatory disease, such as asthma. Further experiments using comprehensive methods such as DNA microarrays and proteomics will be required to conclusively resolve issues of specificity of both siRNA and EGS in specific in vivo applications.

Limited in vitro evidence suggests siRNA appear equipotent to EGS if one can design the EGS based on RNase accessibility. However, an EGS targeted to an identical site as siRNA may or may not be as potent. This is due primarily to the accessibility of the EGS to its target site. For RISC, these rules are less well understood because of the involvement of helicases which interact with RISC and functions in the modulation of the secondary structure of the target mRNA and/or RISC loading (Bernstein et al 2001; Robb

and Rana 2007). The availability of premade cocktails of siRNA as well as the widespread use of siRNA render this the method of choice for gene expression silencing in life sciences discovery. However, in many applications, it is also preferable to use a second, independent method, to validate results. In this context, EGS may be particularly useful given its specificity and relative ease of design and use. Finally, EGS may be the tool of choice where effective and specific siRNAs cannot be designed.

Once a target is validated, EGS can be optimized through unbiased randomization procedures leading to perhaps 100-fold or more increased potency against a specified mRNA target with only small changes in the EGS sequence (Yang et al 2006; Zhou et al 2002). EGS optimization would not be expected to increase non-specific inflammatory and/or pro-apoptotic responses in vivo however, this remains to be tested. siRNA, in contrast, cannot be optimized beyond the point at which the enzyme Slicer, a component of RISC, becomes rate-limiting (Koller et al 2006). In the context of influenza treatment, inflammation may have a beneficial additive or synergistic effect with siRNA but could also exert detrimental effects leading to the development of acute lung injury (ALI) or adult respiratory distress syndrome (ARDS) in the case of reassorted zoonotic strains. Additionally, post-viral exacerbations such as asthma may also be promoted by siRNA induced inflammatory responses. It would probably be most fair to say that the relative potency and specificity of EGS vs siRNA in vivo for treatment of disease cannot be determined on theoretical grounds because of the many interacting and unknown variables, but will require direct comparisons of the two therapies against a particular target when this is possible. It is certainly plausible that there will be some genes or tissues for which EGS silencing is more potent and specific in vivo, and other genes for which the opposite is found, which would suggest a rationale for using both drugs to target a gene of interest in therapeutic applications.

A clear advantage of siRNA is the ability to inactivate viruses such as the respiratory pathogens RSV (Respiratory Syncytial Virus) and rhinovirus that replicate in the cell cytoplasm (Phipps et al 2004; Strayer et al 2005; Kong et al 2007). Since RNase P is only present in the cell nucleus, pathogens whose transcripts reside exclusively in the cell cytoplasm cannot be targeted directly. However, a number of viruses and intracellular pathogens rely on host proteins for key steps in their life cycle (Elliott et al 2007; Fauci 1996; Fauci and Challberg 2005; Gu et al 2004). Where the identity of such proteins is known, it may be possible to target these using EGS. Furthermore, the pathogenicity of these viruses may

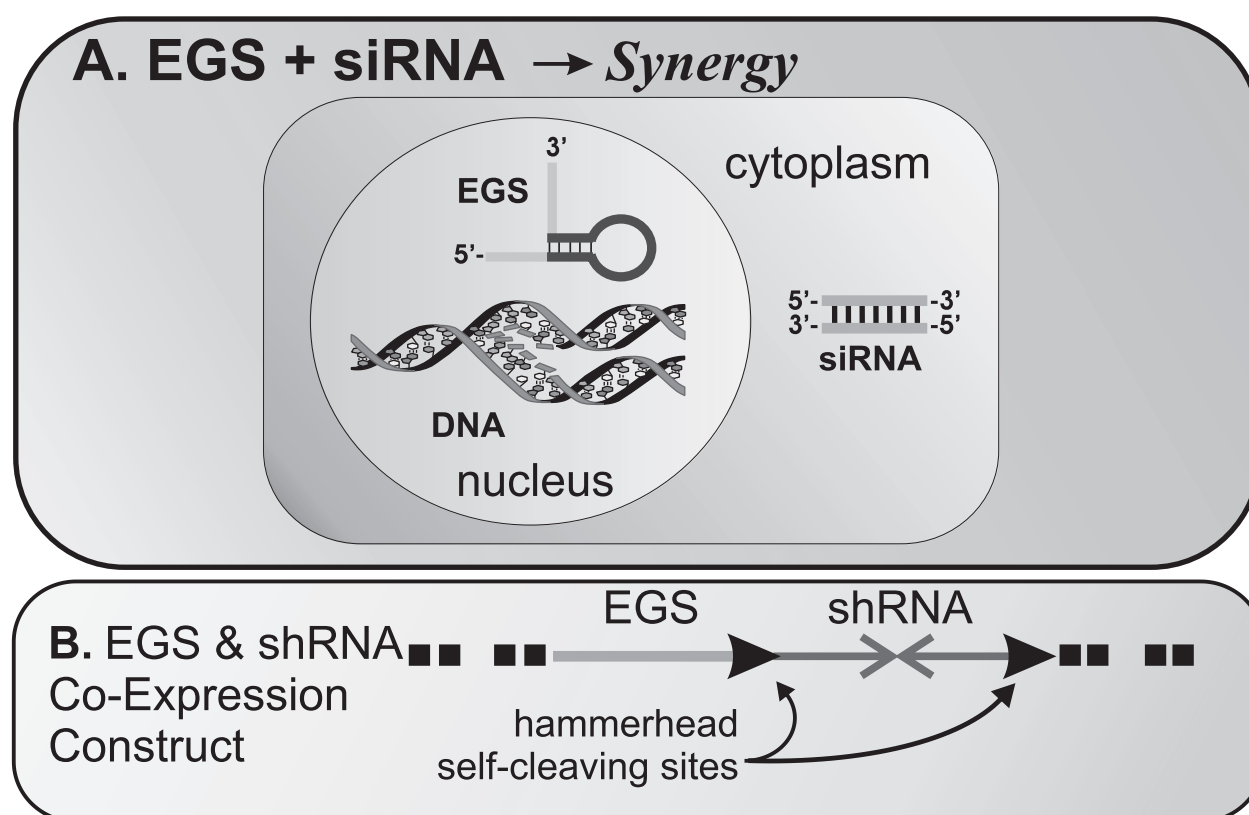


occur through induction of cellular inflammatory cytokines such as interferon or IL-13 (Liu et al 2005); inactivation of these virally triggered cytokines by EGS or inhibition of the NF- $\kappa$ B transcription factor which acts as a master switch, might still be an effective immunotherapy for these cytoplasmic pathogens. Using EGS, it may be possible to differentially inactivate specific subunits of NF- $\kappa$ B which would still allow other subunits to engage in transcriptional activation. Alternatively, the receptors for the pathogens could also be silenced with EGS (Barth, Liang et al 2006; Barth, Schnober et al 2006; Julg and Goebel 2005; Weber et al 2006; Lederman et al 2006), preventing infection or reducing spread and virus load in an ongoing infection. Since the pathogenesis of influenza as well as RSV involves modulation of cytokines such as decreased expression of interferon, and increased synthesis of IL-4 and IL-13 that play a role in post viral exacerbations of asthma (Elliott et al 2007; Oh et al 2002; Minor et al 1976; Singh and Busse 2006; Tekkanat et al 2001; Wang et al 2004; Yasuda et al 2005), it is likely that a combination of anti-viral and anti-cytokine therapies may be required for efficacy in both influenza and post viral asthma.

Perhaps the most intriguing possibility is that EGS and siRNA gene silencing may synergize since they use different mechanisms to inactivate target mRNA yet utilize similar or identical methods of delivery. Figure 4 depicts a scheme by which synergy might be attained by the co-expression of EGS and siRNA if expressed from DNA or EGS and siRNA if administered as RNA oligonucleotides. Advances in delivery of one modality will almost certainly benefit the delivery of the other in a particular disease process. We hypothesize that for pharmacological applications against human respiratory diseases such as influenza and asthma, as well as other diseases which are accessible to either modality, some combination of EGS and siRNA may be optimal in terms of onset of action, specificity, and potency.

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**Figure 4** (A) Potential for synergy between EGS and siRNA: Co-expression of EGS in the nucleus and siRNA in the cytoplasm could effectively synergize to promote gene expression silencing since the two use different pathways. (B) Stable transfection of cDNA constructs encoding EGS and shRNA could also achieve the same results – as shown, we propose a self-cleaving transcript using hammerhead ribozymes encoded in the flanking regions of the shRNA sequences.

at the Yale University School of Medicine, Department of Pediatrics, New Haven, CT and is also the founder and Medical Director of Keren Pharmaceutical, Inc. a biotechnology company focused on the use of EGS technology in human therapeutics and research applications. Dr Fuleihan's present address is the Department of Pediatrics, Children's Memorial Hospital, Chicago, IL (r-fuleihan@northwestern.edu). Dr S Mark Tompkins is an Assistant Professor in the Department of Infectious Diseases, at the University of Georgia College of Veterinary Medicine and is a consultant to Keren Pharmaceutical. Dr Lucy Y Ghoda, is an Investigator at the Webb-Waring Institute, and is a faculty member in the Division of Pulmonary Sciences, Department of Medicine, School of Medicine, University of Colorado Health Sciences Center. She is Scientific Director of Keren Pharmaceutical.

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