Small Interfering RNA

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BACKGROUND: PROCESSES OF RNA INTERFERENCE

Initially postulated by Francis Crick, the central dogma of molecular biology outlines the flow of genetic information through transcription of double-stranded DNA into single-stranded messenger RNA (mRNA), followed by translation into proteins, yielding the building blocks of life. The processes of transcription and translation are regulated by numerous molecular pathways, resulting in complex and sophisticated modifications to this linear process from gene to protein. However, in the late 1990s, researchers demonstrated the ability of certain RNA molecules to reduce the expression of particular genes through a process now collectively termed RNA interference (RNAi) (Fire *et al.*, 1998). Since its initial discovery in the laboratory worm *Caenorhabditis elegans*, small interfering RNA (siRNA) has been used to impact gene expression in research laboratories around the world as an extraordinarily powerful genetic tool in biology and medicine for the elucidation of molecular pathways in organismal development and human disease.

RNAi takes advantage of the fundamental principle of complementary base pairing between nucleic acids, such as in DNA–DNA and RNA–DNA double helices. In principle, RNAi is a natural process within cells, wherein double-stranded microRNA transcripts are expressed and participate in gene regulation. In addition, experimental cellular manipulation of double-stranded RNA serving as siRNAs of 20 to 30 base pairs in length (Meister and Tuschl, 2004) can be designed according to the complementarity principle to specifically target a particular gene's mRNA transcript. Following the same mechanism as microRNA-mediated silencing, the experimentally introduced double-stranded RNAs are processed by a member of the Dicer enzyme family of RNA endonucleases, which trim the double-stranded RNAs into fragments of 21 to 23 base pairs in length. These double-stranded RNAs are then unwound into two short, single-stranded siRNAs (the leading and the lagging strands). The lagging strand is degraded intracellularly, whereas the leading strand binds the multicomponent protein complex termed the RNA-induced silencing complex (RISC) in the cellular cytoplasm. When the RNAi-loaded RISC complex comes into contact with the complementary target gene mRNA transcript, base pairing occurs. This base pairing activates the cleavage mechanism of the RISC complex and is catalyzed by a member of the Argonaute protein family. The target mRNA transcript is cleaved, rendering it untranslatable, and, hence, synthesis of the particular protein synthesis is prevented. Given its genetic mechanism of action, siRNA is considered a powerful technique for posttranscriptional gene silencing. The regulation of siRNA-mediated RNAi is an area of ongoing research.

WHAT IS siRNA?

- Small interfering RNA (siRNA) represents a form of posttranscriptional gene silencing.
- siRNAs are designed to bind specific mRNA molecules and target them for degradation.
- Common targets of siRNAs include mRNA transcripts of mutant genes underlying genetic disorders, as well as normal genes involved in the cell replication process that are overexpressed in cancer cells.

LIMITATIONS

- Delivery of siRNAs into cells *in vitro* by transfection is a complex process with variable success.
- Translating siRNAs for therapeutic purposes into humans is complicated by factors of appropriate delivery to target cells and stimulation of host immune responses.
- Measuring and quantifying *in vivo* effects of therapeutic siRNA are challenging.

TECHNICAL ASPECTS OF siRNA

Approaching genetic silencing using siRNA requires knowledge of the target gene's gene sequence and specifically its spliced mRNA transcript that is the intended target. A series of siRNAs complementary to regions of the target mRNA transcript should be designed and assessed for their individual efficacy. When using siRNA as a proof-of-function or therapeutic strategy for genetic diseases, researchers tend to identify unique sequences within the target mRNA responsible for aberrant function (such as the site of a mutant allele) as a template by which to design siRNA with specific degradation ability.

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Once the siRNAs are available as double-stranded RNAs following their chemical synthesis, they must then be introduced into the cells of interest via a process known as transfection. Various methods of transfection are available; a common technique involves mixing siRNA with chemical lipids to form liposomes, which enables relatively effective delivery through cell membranes into the cytoplasm. Once the siRNAs are introduced into the cellular cytoplasm, they are processed by cellular enzymes as described above and can mediate posttranscriptional gene silencing. A major challenge to siRNA effectiveness relates to the cytoplasmic degradation of siRNAs that can be delayed by chemical modification of the synthesized RNA. Quantification gene silencing efficacy can be measured using several techniques, including real-time quantitative PCR (qPCR) to assess target mRNA transcript levels (Garibyan and Avashia, 2013) and Western blotting analysis to address effects on protein levels (Nicholas and Nelson, 2013). The methodology of siRNA-mediated RNAi is summarized in Figure 1.

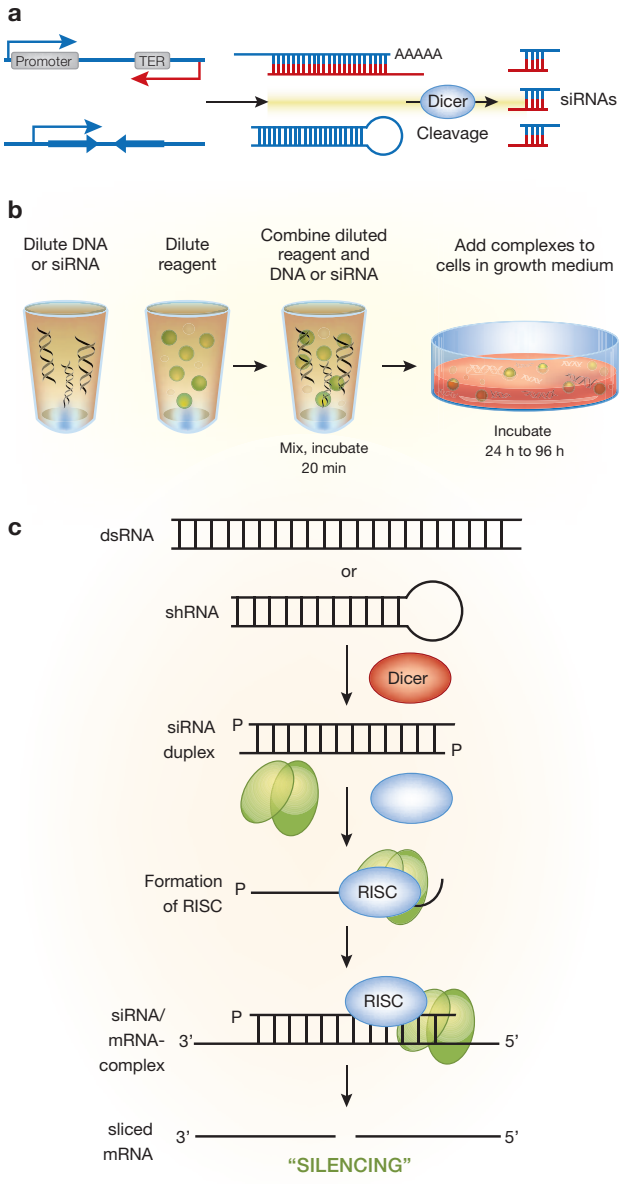

Figure 1. Summary of steps of the siRNA lab technique and intracellular mechanism. (**a**) Identification of target gene DNA leads to design of complementary siRNA that will bind to a DNA target site. (**b**) Transfection of siRNA into cells using liposomal reagents. (**c**) Intracellular mechanism of siRNA.

APPLICATIONS OF SIRNA IN DERMATOLOGY RESEARCH

The advantage of siRNA relies upon gene-specific downregulation to target skin disorders and genodermatoses caused by a single dominant gene. Recently, Pendaries and colleagues studied the potential of siRNA as a modality to inhibit abnormal collagen synthesis in a cellular model of dominant dystrophic epidermolysis bullosa (DDEB) (Pendaries *et al.*, 2012). DDEB, also known as Cockayne–Touraine disease, is caused by mutations in the gene *COL7A1* encoding a mutant type VII collagen α1 subunit that fail to assemble appropriately, resulting in generalized persistent blistering of the skin. Pendaries and colleagues designed 21 distinct siRNAs targeted against a specific *COL7A1* mutation—deletion of exon 87—associated with DDEB pruriginosa. The siRNAs were designed to interfere with the mutant sequence formed at the aberrant splice junction of the defective gene. Using siRNAs that were delivered into cultured cells with the mutant *COL7A1* gene as well as normal cells (Figure 2a and 2b), 11 of the siRNAs were able to achieve >40% inhibition of mutant *COL7A1* RNA levels. The specificity of these siRNAs for downregulating mutant gene expression was further studied by evaluating their effectiveness in fibroblasts derived from DDEB pruriginosa patients in comparison to healthy (normal) control patients, demonstrating 40–60% reductions in mutant gene expression, with negligible inhibition of wild-type collagen VII RNA levels (Figure 3). These findings, in conjunction with similar reports using allele-specific siRNA targeted against keratin 5 mutations in epidermolysis bullosa simplex (Atkinson *et al.*, 2011), offer a glimpse of future clinical applications of siRNA techniques in the management of chronic blistering skin disease.

In addition to blistering skin diseases, siRNA has been studied as a potential therapeutic strategy in a variety of other cutaneous conditions, including skin cancers. Because tumor cells exhibit higher rates of cell proliferation than normal cells, siRNAs may be used to target critical components of cell replication. In 2010, Zuckerman and colleagues examined the M2 subunit of ribonucleotide reductase (RRM2)—a key protein in DNA replication, required for cell proliferation—as a target for siRNAs in melanoma cells (Zuckerman *et al.*, 2010). In Figure 4, the authors demonstrate the efficacy of the targeted siRNA, termed siR2B+5, at decreasing the expression of both RRM2 mRNA and protein relative to control siRNA, as evidenced by qPCR and Western blot analysis. The authors confirmed that this siRNA interference functionally affected cell proliferation by demonstrating decreased cell viability in a variety of melanoma cell lines cultured *in vitro* (Figure 5a) and a dose-dependent response (Figure 5b). Finally, the authors were able to show that cotreatment of melanoma cell lines with siRNA and temozolomide—a chemotherapeutic DNA alkylating agent that

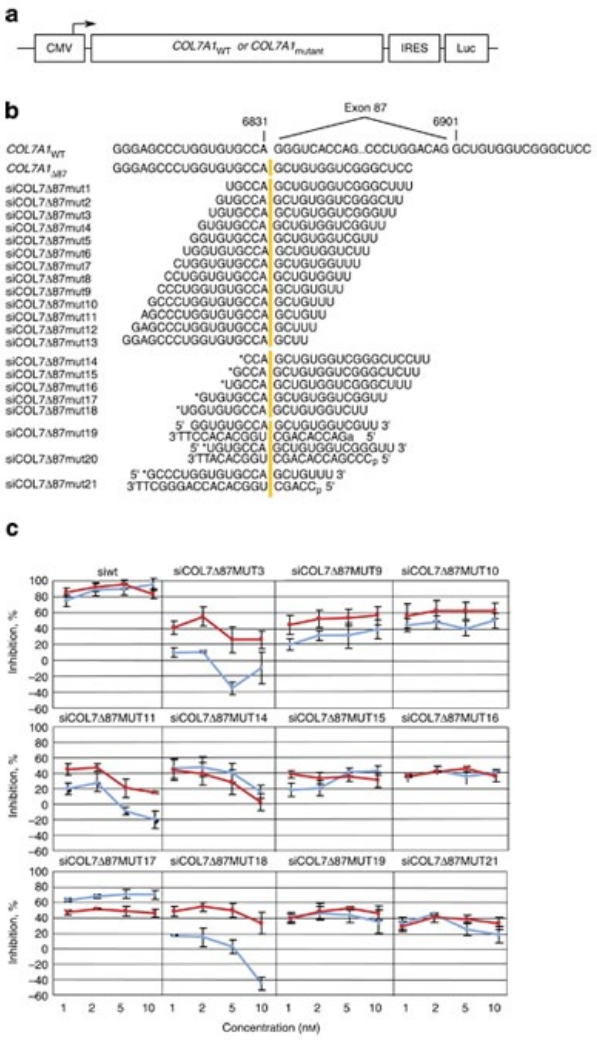

Figure 2. Assay for COL7A1 mRNA inhibition by siRNAs. (**a**) The structural elements of the *COL7A1* gene introduced into the cells. (**b**) The base-pair structures of siRNAs that were studied for silencing the mutant *COL7A1* gene in DDEB. (**c**) The efficacy of the 11 siRNAs that demonstrated over 40% inhibition of mutant gene expression. Reprinted from Pendaries *et al*. (2012).

interrupts DNA replication—resulted in enhanced antitumor effects that were greater than that of either agent alone (Figure 6). Other researchers have used siRNA conjugated with anti-tumor nanoparticles to demonstrate efficacy against melanoma cells *in vitro* (Chen *et al*., 2010). Similar applications of siRNA in combination with nanoparticle-deliverable chemotherapeutic agents directed against melanoma may show promise as a novel means to achieve tumor-targeted treatment in the future.

FUTURE DIRECTIONS

The past decade has seen tremendous growth and use of siRNA techniques for gene silencing and targeted drug delivery. Such laboratory insights have led to early clinical trials seeking to translate these bench applications to the bedside. However, several limitations of delivering siRNAs therapeutically in living organisms exist (Pecot *et al*., 2011). Biological barriers include stabilizing siRNAs for intravascular delivery,

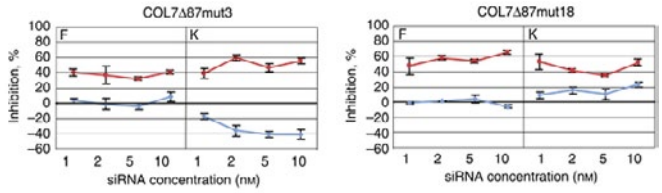

Figure 3. Allele-specific inhibition of endogenous *COL7A1Δ87* mRNA. Significant gene silencing by siRNAs of mutant type VII collagen in fibroblasts derived from patients with dominant dystrophic epidermolysis bullosa (red line) versus fibroblasts derived from healthy control subjects (blue line). Adapted from Pendaries *et al*. (2012).

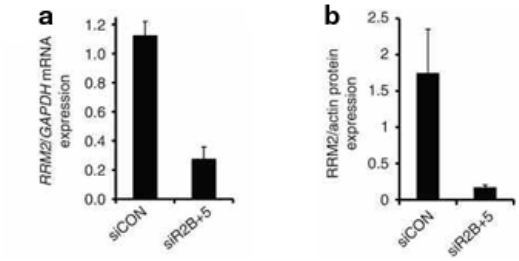

Figure 4. Knockdown of ribonucleotide reductase subunit-2 (RRM2) mRNA and protein expression by siRNA siR2B+5 in melanoma cells. (**a** and **b**) The efficacy of siR2B+5, a targeted siRNA directed against the mRNA encoding the M2 subunit of ribonucleotide reductase (RRM2), at decreasing the expression of both the target mRNA (**a**) and the RRM2 protein (**b**) levels relative to control siRNA. Adapted from Zuckerman *et al*. (2010).

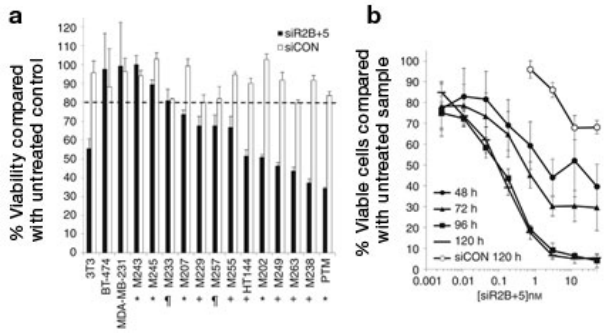

Figure 5. *In vitro* antiproliferative effects of ribonucleotide reductase subunit-2 knockdown by siR2B+5 siRNA in a panel of melanoma cell lines. (**a**) Decreased cell viability in melanoma cell lines transfected with siR2B+5 siRNA versus control siRNA. (**b**) A dose response in cell viability over time with increasing concentrations of siRNA resulting in decreased viability. Reprinted from Zuckerman *et al*. (2010).

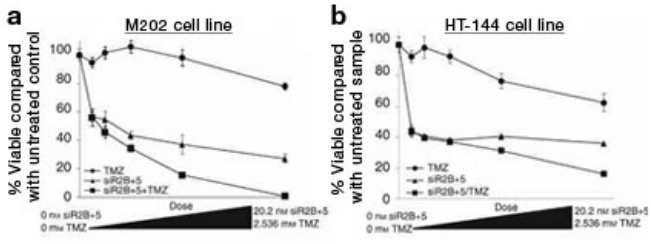

Figure 6. Synergy between siR2B+5 and temozolomide treatment. (**a** and **b**) Cotreatment of two distinct melanoma cell lines (M202 and HT-144) with both siRNA and temozolomide had an antitumor effect that was greater than that of either agent alone. Adapted from Zuckerman *et al*. (2010).

achieving efficient target tissue penetrance, and limiting off-target effects of the siRNAs. One significant challenge is limiting host immune recognition of siRNAs resulting in stimulation of an inflammatory response; techniques to modify siRNAs to adapt to these constraints are currently being developed.

Despite these challenges, application of siRNAs to *in vivo* treatment of human disease has already begun. Phase I clinical trials of systemically delivered siRNA using nanoparticles to target melanoma have demonstrated early promise (Davis *et al*., 2010). Trials using intradermal injection of siRNAs developed for the treatment of pachyonychia congenita (Smith *et al*., 2008; Leachman *et al*., 2010) have similarly been initiated in patient groups. One limitation of siRNA-based RNAi in clinical trials is accurately assessing efficacy on the molecular level. Strategies using qPCR from skin samples are one avenue of measurement, but definitive end points for patient surveillance remain to be elucidated (Hickerson *et al*., 2010). Although still an emerging technique, achieving RNAi via siRNAs has already captured the attention of researchers and clinicians alike in achieving better understanding and more effective therapeutics for a spectrum of cutaneous disorders.

CONFLICT OF INTEREST

RC has received stock options from TremRx resulting from an IP license. The other authors state no conflict of interest.

CME ACCREDITATION

This CME activity has been planned and implemented in accordance with the Essential Areas and Policies of the Accreditation Council for Continuing Medical Education through the Joint Sponsorship of ScientiaCME and Educational Review Systems. ScientiaCME is accredited by the ACCME to provide continuing medical education for physicians. ScientiaCME designates this educational activity for a maximum of one (1) AMA PRA Category 1 Credit™. Physicians should claim only credit commensurate with the extent of their participation in the activity.

To take the online quiz, follow the link below:
http://www.classmarker.com/online-test/start/?quiz=ej75241ca0f241ac

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SUPPLEMENTARY MATERIAL

A PowerPoint slide presentation appropriate for journal club or other teaching exercises is available at http://dx.doi.org/10.1038/jid.2013.411.

QUESTIONS

This article has been approved for 1 hour of Category 1 CME credit. To take the quiz, with or without CME credit, follow the link under the "CME ACCREDITATION" heading.

1. The typical length of siRNAs used for gene silencing is:

A. 7 to 9 base pairs.

B. 21 to 23 base pairs.

C. 36 to 38 base pairs.

D. 55 to 57 base pairs.

2. siRNAs are considered what form of gene expression modification?

A. Transposon-mediated gene silencing.

B. Histone-mediated gene silencing.

C. Posttranscriptional gene silencing.

D. Posttranslational gene silencing.

3. The process of introducing siRNAs into cells is known as:

A. Transposition.

B. Translation.

C. Translocation.

D. Transfection.

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