(twice-weekly) treatment resulted in nearly 90% survival in the 1-month study, with essentially normalized lung histology and lung function. The authors emphasize that modification of mRNA substantially reduced the amount of cytokines (IFN-γ and IL-12) measured in bronchial lavage fluids soon (8 hours) after administration.

The development of mRNA as a therapeutic faces the same challenge as any nucleic acid: delivery, delivery, delivery. Extension of the approach described by Kormann *et al.*[1] to other targets will probably require some degree of compaction and complex formation. On the other hand, the effectiveness of intravascular delivery under pressure ("hydrodynamic" delivery) of uncomplexed DNA might extend to modified mRNA delivery into liver,[8,9] muscle,[10] and other organs. One limitation of modified mRNA delivery compared with integrative gene transfer[11] is the transient duration of expression, as exemplified in the Epo studies that Kormann *et al.* report. Moreover, erythropoiesis is exquisitely sensitive to even slight increases of Epo in the circulation. For many other therapeutic gene products, such as clotting factors, enzymes, and even cytokines, a much greater amount of gene product will be necessary. It is therefore likely that substantial improvements will be required in the efficiency of mRNA delivery and translation into protein product to reach a level that is of more general therapeutic utility.

In these days of heightened safety concerns, it seems as though every advance in gene transfer and expression needs to somehow provide a solution to the problem of leukemogenesis.[12] Therefore, one of the incentives for testing the *in vivo* effectiveness of modified RNA was to provide an alternative to the potential risk of insertional mutagenesis associated with integrative DNA gene transfer and expression. However, adverse events associated with integrative gene transfer have thus far been limited to circumstances in which continued expression of the gene product is required in cellular progeny after extensive proliferation and differentiation.[13] Modified mRNA is unlikely to be maintained at a level sufficient for corrective expression of gene product in these circumstances, so alternative means of supporting maintained expression (i.e., corrective gene integration or chromosomal modification) will be required in these cases.

At present, applications of this technology will include those in which a short or intermittent burst of gene product is anticipated to have a beneficial effect. This extends beyond protein replacement—for example, in the use of modified mRNA for reprogramming in stem cell generation and differentiation.[14] Modified mRNA may also be used to express a recombinase that mediates genome modification[15,16] or nucleases for site-specific chromosomal modification,[17] because a short duration of expression may be sufficient while avoiding unwanted gene integration and long-term expression. The results reported by Kormann *et al.* thus provide new inspiration for the therapeutic testing of mRNA.

REFERENCES

1. Kormann, MS, Hasenpusch, G, Aneja, MK, Nica, G, Flemmer, AW, Herber-Jonat, S *et al.* (2011). Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. *Nat Biotechnol* **29**: 154-157.
2. Hannon, GJ and Rossi, JJ (2004). Unlocking the potential of the human genome with RNA interference. *Nature* **431**: 371-378.
3. Weide, B, Pascolo, S, Scheel, B, Derhovanessian, E, Pflugfelder, A, Eigentler, TK *et al.* (2009). Direct injection of protamine-protected mRNA: results of a phase 1/2 vaccination trial in metastatic melanoma patients. *J Immunother* **32**: 498-507.
4. Cannon, G and Weissman, D (2002). RNA based vaccines. *DNA Cell Biol* **21**: 953-961.
5. Kawai, T and Akira, S (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* **11**: 373-384.
6. Brennan, K and Bowie, AG (2010). Activation of host pattern recognition receptors by viruses. *Curr Opin Microbiol* **13**: 503-507.
7. Kariko, K, Buckstein, M, Ni, H and Weissman, D (2005). Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity* **23**: 165-175.
8. Liu, F, Song, Y and Liu, D (1999). Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther* **6**: 1258-1266.
9. Zhang, G, Budker, V and Wolff, JA (1999). High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum Gene Ther* **10**: 1735-1737.
10. Zhang, G, Wooddell, DI, Hegge, JO, Griffin, JB, Huss, T, Braun, S *et al.* (2010). Functional efficacy of dystrophin expression from plasmids delivered to mdx mice by hydrodynamic limb vein injection. *Hum Gene Ther* **21**: 221-237.
11. Score, PR, Belur, LR, Frandsen, JL, Geurts, JL, Yamaguchi, T, Somia, NV *et al.* (2006). *Sleeping Beauty*-mediated transposition and long-term expression *in vivo*: use of the LoxP/Cre recombinase system to distinguish transposition-specific expression. *Mol Ther* **13**: 617-624.
12. Hacein-Bey-Abina, S, Von Kalle, C, Schmidt, M, McCormack, MP, Wulffraat, N, Leboulch, P *et al.* (2003). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**: 415-419.
13. Qasim, W, Gaspar, HB and Thrasher, AJ (2009). Progress and prospects: gene therapy for inherited immunodeficiencies. *Gene Ther* **16**: 1285-1291.
14. Warren, L, Manos, PD, Ahfeldt, T, Loh, Y-H, Li, H, Lau, F *et al.* (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* **7**: 618-630.
15. Wilber, A, Frandsen, JL, Geurts, JO, Largaespada, DA, Hackett, PB, McIvor, RS (2006). RNA as a source of transposase for *Sleeping Beauty*-mediated gene insertion and expression in somatic cells and tissues. *Mol Ther* **13**: 625-639.
16. Wilber, A, Wangensteen, KJ, Chen, Y, Zhuo, L, Frandsen, JO, Bell, JB *et al.* (2007). Messenger RNA as a source of transposase for *Sleeping Beauty* transposon-mediated correction of hereditary tyrosinemia type I. *Mol Ther* **15**: 1280-1287.
17. Urnov, FD, Rebar, EJ, Holmes, MC, Zhang, HS and Gregory, PD (2010). Genome editing with engineered zinc finger nucleases. *Nat Rev Genet* **11**: 636-646.

Sensor and Sensitivity: A Screen for Elite shRNAs

Daniela Castanotto[1]

doi:10.1038/mt.2011.70

Creation of inhibitors targeted to a gene of interest is key both to the study of gene function and to drug development, and RNA interference (RNAi) is an attractive approach that complements the development of standard small-molecule drugs. A key factor for the successful application of this technology is the design of potent triggers of RNAi that can be employed at sufficiently low concentrations to avoid or minimize off-target or toxic effects. In an article that appeared recently in *Molecular Cell*,[1] Fellmann *et al.* report the development of a library selection screen

[1] *Division of Molecular Biology, Beckman Research Institute of the City of Hope, Duarte, California USA*

Correspondence: *Daniela Castanotto, Division of Molecular Biology, Beckman Research Institute of the City of Hope, 1500 Duarte Road, Duarte, California 91010, USA. E-mail: DCastanotto@coh.org*

© The American Society of Gene & Cell Therapy

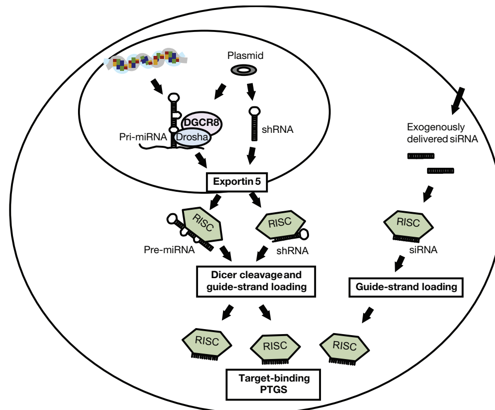

Figure 1 Schematic representation of RNA interference mechanisms. Primary endogenous or recombinant miRNAs (pri-miRNAs) are processed by Drosha and its partner DGCR8 to ~70-nt precursors, which are transported to the cytoplasm by Exportin 5. Plasmid-expressed short hairpin RNAs (shRNAs) do not require Drosha processing and use the same export pathway to reach the cytoplasm. Following Dicer cleavage, which yields short 21- to 23-nt double-stranded RNA molecules, the functional or "guide" strand is loaded into the RNA-induced silencing complex (RISC). Exogenous 21- to 23-nt small interfering RNAs (siRNAs) are delivered into the cytoplasm and directly loaded into the RISC. The RISC-bound guide strand is directed to bind to its target and triggers posttranscriptional gene silencing (PTGS).

for the generation of potent RNAi triggers that is likely to work for virtually any target gene transcript. The selection method screens every possible overlapping sequence stretch for a particular target gene, thereby providing complete coverage of a given target to identify the most efficacious RNAi triggers.

RNAi is based on a ubiquitous mechanism of gene regulation in eukaryotic cells mediated by endogenous microRNAs (miRNAs). The process triggers sequence-specific RNA degradation resulting in posttranscriptional silencing of targeted genes. RNAi can also be triggered by the delivery of small interfering RNAs (siRNAs) directly to cells or tissues or by a gene therapy approach that expresses short hairpin RNAs (shRNAs) from plasmid or viral vectors. Primary endogenous miRNA transcripts are processed to precursor miRNAs of approximately 70 nucleotides (nt), which in turn are processed by the enzyme Dicer into double-stranded 21- to 23-nt miRNAs[2], whereas shRNAs expressed from gene vectors are directly converted into siRNAs by Dicer processing. The functional, or "guide," strand of these siRNAs is then loaded into the RNA-induced silencing complex (RISC), which guides it to the complementary target site and catalyzes the cleavage and degradation of the target messenger RNA (mRNA; **Figure 1**).

Rules for the generation of effective shRNAs and siRNAs have emerged in the past several years[3] and have been incorporated into algorithms that take into account secondary structural features of the target RNAs and the thermodynamic stability of the siRNAs' ends. Although these algorithms can help identify effective synthetic siRNAs,[4,5] they are often not useful for identifying optimal shRNAs because we still do not fully understand the biology of RNAi guide-strand selection.[6,7] Another important consideration when designing RNAi triggers is that exogenously delivered siRNAs or shRNAs act via the same cellular pathways as endogenous miRNAs, posing the risk of saturating essential components of the RNAi machinery. Indeed, competition for incorporation into the RISC can lead to cellular toxicity.[8] Thus, it is important to identify the most potent siRNA or shRNA that can be effective at the lowest possible concentration. shRNA or siRNA asymmetry is another important parameter because the relative ability of the guide strand to be selected and incorporated into the RISC over the so-called "passenger" strand is a key consideration in avoiding the risk of off-target effects. Some modifications of the passenger strand can be incorporated to preferentially facilitate incorporation of the guide sequence,[9,10] but this is feasible only for the design of synthetic siRNAs. Ultimately, the best siRNA or shRNA will be determined by a combination of factors, including specific nucleotides at set positions, asymmetry, and target accessibility. However, it is not currently possible to predict the efficacy of a designed siRNA or shRNA without empirical testing. Thus, the identification of truly potent shRNAs is not a trivial task, and the development of a robust approach for the design of expressed shRNAs is an important advance in the field.

Genome-wide siRNA and shRNA libraries, those focused on subsets of genes involved in specific cellular pathways, and custom libraries are commercially available for use in screening. However, they carry the inherent downside of a possible lack of target specificity because not all siRNAs or shRNAs that favor incorporation of the passenger strand are excluded. Moreover, libraries usually do not include every possible siRNA or shRNA design and carry the risk of missing a potent inhibitor of some cellular targets. To address these issues, Fellmann and colleagues[1] tiled nine different mammalian transcripts with a library of 20,000 shRNAs directed against every possible target site in these mRNAs, using siRNAs that cover bases 1-21, 2-22, 3-23, etc. The shRNAs were inserted into a miRNA scaffold so as to undergo miRNA pathway processing, which has been shown to minimize competition with endogenous miRNAs by the exogenous sequences.[11] To express the shRNA sequences, they were placed under the control of a doxycycline-inducible promoter in a viral vector that also contained the target (or sensor) gene of the shRNA fused to a green fluorescent reporter gene, *Venus*. Thus, shRNAs that effectively inhibited the expression of their cognate targets in the sensor would also inhibit expression of the reporter gene, resulting in loss of fluorescence (**Figure 2**). Repetitive rounds of selection involving induction and withdrawal of doxycycline, and hence both up- and downregulation of the shRNAs, were accompanied by fluorescence-activated cell sorting. This "ping-pong" strategy allowed the gating and identification of the best shRNA and target site combinations for each gene. The most potent shRNAs showed a strong bias for incorporation of the intended guide strand (target complementary sequence) into the RISC. An interesting result of these analyses is that

potent, single-copy shRNAs were found to be rather uncommon, representing only 2.4% of all the possible shRNAs.

The analyses of the selected shRNA-guide sequence combinations are consistent with previously established rules for siRNA design.[3] The most effective siRNAs and shRNAs contained 9-18 A/U nt and a low G/C content. As has been found previously for the design of siRNAs, the authors determined the advantage of an A/U-rich 5′ end of the antisense strand, an A/U at position 10, thermodynamic asymmetry, and a lack of internal repeats.[3] An A/U at position 13 and a U at position 14 were also consistent with previous findings.[3] Fellmann *et al.* also showed that an A/U at position 13 or 14, a G at position 20 or 21, and a C at position 20 affected the accuracy of cleavage of primary or recombinant endogenous miRNAs (pri-miRNAs). Including these newly identified rules in the design of miRNA scaffolds for shRNA expression will ensure more efficient processing and thus greater potency of the mature siRNA sequence. Moreover, the effect of asymmetry that has previously been implicated in RISC loading seems instead to be more important at a later step—when the siRNA guide sequence interacts with its mRNA target—perhaps to facilitate unwinding and RISC turnover.[12,13] Finally, although it has long been thought that noncoding or regulatory regions should be avoided because protein binding or secondary structures within these regions might impair siRNA target pairing, the authors showed that, with the exception of G/C-rich regions, targets for potent shRNAs seem to be evenly distributed throughout the transcripts.

Although a portion of the shRNA library was not processed according to the previously known structure and processing requirements for miRNAs, which is important in the context that Dicer and RISC association with processed siRNAs can differ dramatically between two siRNAs differing by only a single nucleotide,[14] this scheme represents the most powerful shRNA selection approach to date because it is able to functionally identify the most optimal shRNA for a given target. Thus, this strategy should become the method of choice for identifying and selecting shRNAs for therapeutic applications, because these must be potent and highly target-specific at the lowest possible concentration. Such a screening system should also be appealing to companies as a high-throughput method for identifying potent shRNAs for virtually any gene of interest. The *Venus* fluorescence marker could also potentially be replaced with a "suicide" gene such as *thymidine kinase* or *cytosine deaminase*. Strong downregulation of the target would result in positive selection in the presence of the pro-drugs ganciclovir or 5-fluorocytosine, which could simplify the selection screen, perhaps also rendering it a more facile approach for use in small academic gene therapy labs.

RNAi continues to show great potential for therapeutic applications, including treatment of cancer as well as cardiovascular, neurological, and metabolic diseases and viral infections.[15,16] However, for it to achieve therapeutic success, its potency and safety must be increased by eliminating the unwanted outcomes of suboptimal RNAi trigger designs. The work by Fellmann and colleagues has brought us one step closer to this ultimate aim.

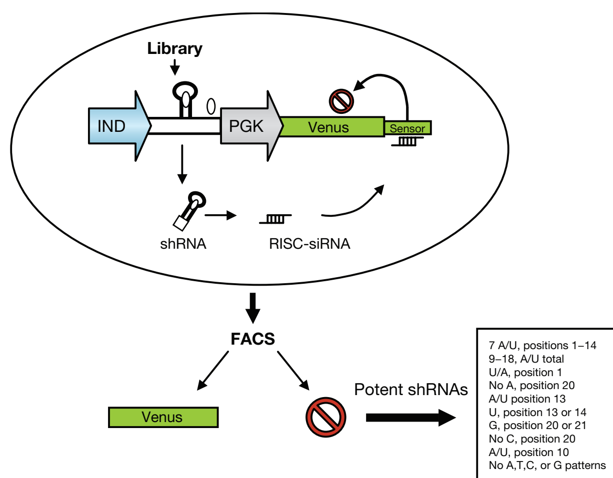

Figure 2 Sensor assay to select potent shRNAs. A library of 20,000 short hairpin RNAs (shRNAs) was cloned into an miR30 scaffold under the control of a Tet-inducible promoter (IND) in a vector (pSensor) expressing a green fluorescent protein (GFP) marker gene (*Venus*) linked to a "sensor" target region containing the cognate 50-nt target region from nine different genes targeted for silencing. Following induction, the shRNAs that are able to inhibit their target will in turn reduce or block GFP expression. The most potent shRNAs can be then isolated following fluorescence-activated cell sorting (FACS) of the cells that lose GFP expression. Nucleotides found at high frequency in the most effective shRNAs are indicated. PGK, phosphoglycerate kinase promoter; RISC, RNA-induced silencing complex; siRNA, small interfering RNA.

REFERENCES

1. Fellmann, C, Zuber, J, McJunkin, K, Chang, K, Malone, CD, Dickins, RA *et al.* (2011). Functional identification of optimized RNAi triggers using a massively parallel sensor assay. *Mol Cell* **41**: 733–746.
2. Newman, MA and Hammond, SM (2010). Emerging paradigms of regulated microRNA processing. *Genes Dev* **24**: 1086–1092.
3. Hajeri, PB and Singh, SK (2009). siRNAs: their potential as therapeutic agent—Part I. Designing of siRNAs. *Drug Discov Today* **14**: 851–858.
4. Huesken, D, Lange, J, Mickanin, C, Weiler, J, Asselbergs, F, Warner, J *et al.* (2005). Design of a genome-wide siRNA library using an artificial neural network. *Nat Biotechnol* **23**: 995–1001.
5. Vert, JP, Foveau, N, Lajaunie, C and Vandenbrouck, Y (2006). An accurate and interpretable model for siRNA efficacy prediction. *BMC Bioinformatics* **7**: 520.
6. Bassik, MC, Lebbink, RJ, Churchman, LS, Ingolia, NT, Patena, W, LeProust, EM *et al.* (2009). Rapid creation and quantitative monitoring of high coverage shRNA libraries. *Nat Methods* **6**: 443–445.
7. Li, L, Lin, X, Khvorova, A, Fesik, SW and Shen, Y (2007). Defining the optimal parameters for hairpin-based knockdown constructs. *RNA* **13**: 1765–1774.
8. Grimm, D, Streetz, KL, Jopling, CL, Storm, TA, Pandey, K, Davis, CR *et al.* (2006). Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* **441**: 537–541.
9. Chen, PY, Weinmann, L, Gaidatzis, D, Pei, Y, Zavolan, M, Tuschi, T *et al.* (2008). Strand-specific 5′-O-methylation of siRNA duplexes controls guide strand selection and targeting specificity. *RNA* **14**: 263–274.
10. Chernolovskaya, EL and Zenkova, MA (2010). Chemical modification of siRNA. *Curr Opin Mol Ther* **12**: 158–167.
11. Castanotto, D, Sakurai, K, Lingeman, R, Li, H, Shively, L, Aagaard, L *et al.* (2007). Combinatorial delivery of small interfering RNAs reduces RNAi efficacy by selective incorporation into RISC. *Nucleic Acids Res* **35**, 5154–5164.
12. Haley, B and Zamore, PD (2004). Kinetic analysis of the RNAi enzyme complex. *Nat Struct Mol Biol* **11**: 599–606.
13. Leuschner, PJ, Ameres, SL, Kueng, S and Martinez, J (2006). Cleavage of the siRNA passenger strand during RISC assembly in human cells. *EMBO Rep* **7**: 314–320.
14. Sakurai, K, Amarzguioui, M, Kim, DH, Alluin, J, Heale, B, Song, MS *et al.* (2011). A role for human Dicer in pre-RISC loading of siRNAs. *Nucleic Acids Res* **39**: 1510-1525.
15. Castanotto, D and Rossi, JJ (2009). The promises and pitfalls of RNA-interference-based therapeutics. *Nature* **457**: 426–433.
16. Lares, MR, Rossi, JJ and Ouellet, DL (2010). RNAi and small interfering RNAs in human disease therapeutic applications. *Trends Biotechnol* **28**: 570–579.