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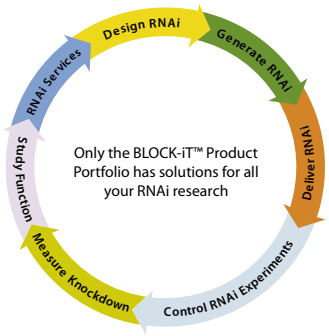

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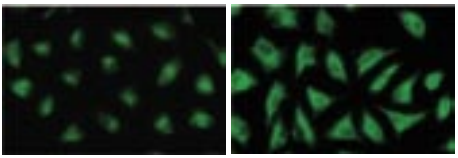

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The importance and impact of RNAi: An introduction by John Rossi, Ph.D.

The discovery of RNA interference (RNAi) has dramatically changed the way most investigators study gene function. First discovered in plants and C. elegans and subsequently shown to be present in mammalian cells as well, RNAi is clearly the most powerful sequence specific inhibitor of gene expression available to investigators. The first demonstrations of RNAi in mammalian cells took advantage of knowledge derived from Drosophila cell extract experiments in which long double stranded RNAs were shown to be degraded by an RNAse III type enzyme into 21-23 nucleotide duplexes with 2 base 3' overhangs. The discovery that these small interfering RNAs (siRNAs) are the triggers for the sequence specific cleavage of RNAs harboring complementary sequences created an entire new industry of synthetic RNA production. The most popular way to trigger RNA in mammalian cells is the use of synthetic siRNAs delivered to cells by cationic lipids. The reason that RNAi is such a potent gene silencing mechanism is that it encompasses the use of a complex of cellular proteins that effectively identify cellular targets with complementary sequences. Of importance to the use of siRNAs is the fact that they can not only trigger complementary sequence degradation, but under certain conditions can also result in sequence specific translational suppression in the absence of target degradation. Most recently it has been demonstrated that siRNAs can also trigger transcriptional gene silencing, although the generality of this phenomenon is yet to be determined.

During a period of only 4 years RNAi has grown from an interesting biological phenomenon to one of the most widely used methods in biology. There is great interest in capturing the power of RNAi for therapeutic use in the treatment of diseases as well. Like any new and powerful technology, the pace of use often exceeds the pace of understanding. This is certainly true for RNAi. There are a growing number of reports demonstrating "off-target" effects elicited by siRNAs as well as activation of the interferon pathway in some cell types. From the therapeutic standpoint, efficacious delivery of siRNAs *in vivo* is the major challenge. Since RNAi is such an important tool for the basic science and pharmaceutical communities, the problems are being addressed as rapidly as they arise. For instance, certain chemical backbone modifications in the siRNAs can minimize if not eliminate "off target" and interferon inducing effects. These same modifications aid in stabilizing siRNAs *in vivo*. There are ingenious applications of fusing siRNAs with targeting ligands or incorporation of siRNAs into nano particles for delivery. Rules for target site selection are becoming more refined. It is clear that this important cellular mechanism for regulating gene expression has become the singular most important tool for the biologist since PCR. Despite this, there is still much to learn about the functional roles small RNAs play in eukaryotic cells. The next few years will certainly provide the scientific community with more exciting discoveries and certainly new applications for RNAi.

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Introduction

RNA interference (RNAi), heralded by *Science* as the Breakthrough of the Year for 2002, seems to be well on its way to becoming a blockbuster research tool as have monoclonal antibodies and PCR.

RNAi is a cellular mechanism that degrades unwanted RNAs in the cytoplasm but not the nucleus. RNAi is also part of the cell's mechanism for defending itself from attack by viruses and potential damage due to jumping genes (transposable elements). The RNAi process has been observed in a wide range of species, including plants, nematodes, protozoa, insects, mammals, and humans.

A powerful research tool for blocking gene expression and assessing gene function has evolved out of plugging into the RNAi pathway thereby leveraging the natural machinery of the cell. By building on the RNAi process, researchers have developed techniques for producing gene-specific loss-of-function or hypomorphic phenotypes. These induced effects are referred to as gene knockdowns since gene function is attentuated, unlike gene knockouts in transgenic animals where the function of the gene is completely lost.

RNAi research has spawn an alphabet soup of acronyms associated with knockdown gene research. Small or short inhibiting RNAs are siRNAs, double-stranded RNAs are dsRNAs. An important enzyme in the process has been dubbed Dicer, as it "dices" longer dsRNAs into shorter siRNAs in the natural cellular mechanism. Other small RNAs are involved in the regulation of cellular processes. These are termed microRNAs (miRNAs) or small temporally regulated RNA (stRNA). miRNAs regulate developmental timing by mediating sequence-specific repression of mRNA translation without affecting mRNA stability in contrast to siRNAs which act as mediators of sequence-specific mRNA degradation.

Discovery of RNAi

More than a decade ago, in an attempt to create altered colors in petunias, researchers discovered a phenomenon similar to what is now termed RNAi while trying to create petunias with increased pigmentation. It was observed that some flowers had decreased pigmentation. In plants, this turning off of genes was originally termed co-suppression. Plants defend against viruses utilizing a mechanism termed post-transcriptional gene silencing (PTGS). In 1998 a discovery, termed RNAi, was found to occur in the nematode worm *C. elegans*. These researchers observed, unexpectedly, that dsRNA was more effective at producing interference than either the sense or antisense strand alone. The first successful reported RNAi in mammalian cells appeared only three years later in 2001. In that first report, transfection of 21-nt siRNA duplexes via cationic liposomes into human embryonic kidney cells and HeLa cells specifically suppressed endogenous and heterologous genes. From this point on the application of RNAi as a research tool has exploded.

Mechanism of Small Interfering RNAs

The dsRNA is processed into shorter units that act to guide the recognition and targeted cleavage of homologous RNA. Triggers for RNAi can be produced in the nucleus or the cytoplasm related to various processes including transcription through inverted DNA repeats, simultaneous synthesis of sense and antisense RNAs, viral replication and the action of cellular or viral RNA-dependent RNA polymerases on single stranded RNA templates. Currently the mechanism of RNAi is thought to be a two-step process. The first step involves a dsRNA ribonuclease-III-like endonuclease activity that processes dsRNA into sense and antisense RNAs 21 to 25 nucleotides in length. These siRNA duplexes have two to three nt 3′ overhanging ends and contain 5′ phosphate and free 3′ hydroxyl termini. An RNase III-type protein termed Dicer is involved in this process.

The second step involves the siRNAs acting as guides for RISC (RNA-induced silencing complex) to cleave the homologous single-stranded mRNAs. Assembly of the RISC complex requires ATP and a single stranded siRNA remains in the RISC complex. RISC cuts the mRNA approximately in the middle of the region paired with the siRNA, after which the mRNA is degraded further.

Genetic studies have revealed nearly a dozen genes that affect the dsRNA response. These genes encode putative nucleases (mut-7), helicases, RNA-dependent RNA polymerases and members of the Argonaute family. A single member of the Argonaute gene family, Ago2, is responsible for mRNA cleavage activity. RNAi and post-transcriptional gene silencing (PTGS) have been shown to protect against invading genetic elements such as transposons, transgenes, and viruses which potentially share a common trigger in the form of a long dsRNA.

RNAi as a Tool

The use of synthetic linear dsRNAs or vector synthesized hairpins can mimick the knockdown response generated by small interfering RNAs created naturally by the enzyme Dicer. Today, reagents such as synthetic RNAs, vectors, and transfection agents are available and have led to the rapid adoption of RNAi as a research tool.

Specificity of RNAi Molecules

Off-target and non-specific RNAi gene knockdown effects may be observed when utililzing RNAi as a tool. In an off-target effect, the sense or antisense strand is completely homologous to another target or has one or a few mistmatches and can still bind and have an effect on an unregulated gene through cleavage or a translational inhibition mechanism. Also, long dsRNA are known to activate various stress response pathways, such as interferon, and ultimately result in apoptosis. With short dsRNA sequences these same types of non-specific effects, can be initiated and lead to global changes in gene expression.

Design of siRNAs

A number of empirical methods have been devised for the design of siRNAs. In one example, the first step in designing an appropriate insert is to choose the siRNA target site. siRNA target sites are typically chosen by scanning an mRNA sequence for AA dinucleotides, recording the 19 nucleotides immediately downstream of the AA, and then comparing the potential siRNA target sequences with an appropriate genome database to eliminate any sequences with significant homology to other genes. Invitrogen and others have developed algorithms that incorporate what is known about the importance of delta G in driving the antisense strand into the RISC. It is known that there are many bases within the sequence which can have individual impact on the ability to knockdown a gene. A better understanding of the importance of each base will improve the design of siRNA's. Today, it is no longer necessary for researchers to test 4 or 5 sequences to find one with good knockdown properties. Newer algorithms are often able to generate a highly effective sequence on the first attempt. Table 1 provides examples of online siRNA design tools available from academic institutions.

Synthesis of dsRNA

dsRNAs for RNAi are often produced by chemical synthesis, by transcription from short DNA templates or by transcription *in vivo* from transfected DNA constructs and siRNA expression vectors.

Table 1. Online RNAi Rational Design Tools

Group	Tool	Location
Hannon lab	RNAi oligo retriever	katahdin.cshl.org:9331/RNAi/html/rnai.html
Tuschl lab	siRNA user guide	www.rockefeller.edu/labheads/tuschl/sirna.html
Whitehead Institute	RNAi selection program	jura.wi.mit.edu/siRNAext/
siRNA database	Published siRNAs	web.mit.edu/mmcmanus/www/siRNADB.html
Max Planck Institute of Molecular Cell Biology and Genetics	Design and Quality Control of RNAi (DEQOR)	cluster-1.mpi-cbg.de/Deqor/deqor.html

Synthetic dsRNA are either in a standard 21 mer dsRNA format with 2 nt overhangs on either end, or as chemically modified synthetic dsRNA molecules that may offer specificity and stability advantages. Recombinant Dicer can also be used to convert large dsRNAs into pools of siRNAs suitable for gene silencing *in vitro*.

Delivery of siRNAs

siRNA molecules are successfully delivered using a number of different methods. The method of delivery depends on whether the siRNAs are for cell-based (*in vitro*) experiments or (*in vivo)* animal experiments. Delivery of siRNAs can be divided into 8 broad categories:

- *Delivery as naked RNA molecules*
- *Delivery via plasmid vectors*
- *Delivery as short hairpin RNAs (shRNAs)*
- *Delivery via lentiviral or other retroviral vectors*
- *Delivery via vectors*
- *Delivery by injection*
- *Delivery by transfection*
- *Delivery in a tissue-specific manner*

For example, in *C. elegans*, the RNA can be injected into the gonad, or introduced by soaking the organism in dsRNA. The first demonstration of RNAi in mammals followed the transfection of synthetic 21-nt siRNA duplexes and reporter vectors into NIH/3T3 (mouse fibroblast), COS-7 (African green monkey kidney), HeLa S3 (human), and 293 (human embryonic kidney) cells.

siRNA Vector Systems

Vector systems have been developed for synthesizing siRNAs or hairpin siRNAs in mammalian cells. In the later case, vectors have also been designed that transcribe siRNAs where one strand can fold back on the other strand in a hairpin structure. The development of hairpins that are active is an efficient way of synthesizing dsRNAs where the functional activity is encoded in one piece of DNA that gets transcribed. Small RNAs are typically synthesized from a DNA template under the control of an RNA polymerase III (Pol III) promoter in transfected cells. Examples of published vectors are summarized in Table 2.

Invitrogen is a provider of vectors for cloning and delivery of RNAi or siRNA.

Viral Vectors

The use of viral vectors can result in the efficient delivery of nucleic acids. Retroviruses, including the lentiviral approach, are the transgene delivery vector of choice as many cell lines are difficult to transfect with plasmid DNA. Oncoretroviruses can only transduce cycling/dividing cells while Lentiviruses can transduce both dividing and non-dividing cells. Recombinant adenoviral vectors have been utilized for the introduction of a siRNA duplex transcribed by two human U6 promoters.

Table 2. Mammalian Cell Vectors

Promoter	Structure	Terminator	Reference
Mouse U6	Tandem	Two U overhang	Yu *et al.* 2002
Human H1 (pSuper)	Hairpin	Two U overhang	Brummelkamp *et al.* 2002a
Human H1 (pRetro-Super-p53)	Hairpin	Two U overhang	Brummelkamp *et al.* 2002b
U6 (pShh1)	Hairpin	Two U overhang	Paddison et. al. 2002
Human U6	Tandem	Two U overhang	Lee *et al.* 2002
Human U6 small nuclear (sn) RNA	Hairpin	Four U overhang	Paul *et al.*2002
Mouse U6	Hairpin	Several U overhang	Sui *et al.* 2002
Human U6	Tandem	Four U overhang	Miyagashi and Taira 2002
Mouse U6	Hairpin, lentivirus	Four U overhang	Rubinson *et al.* 2003
tRNA(Val)	Hairpin	Several U overhang	Kawasaki and Taira 2003
Human U6	Hairpin, lentivirus, retrovirus	Four U overhang	Stewart *et al.* 2003
Human U6	Tandem,adenovirus	Five U overhang	Uchida *et al.* 2004
Human U6	Hairpin, doxycycline-inducible	Five U overhang	Chen *et al.*2003
Human H1	Hairpin, lentivirus, doxycycline inducible	Several U overhang	Wiznerowicz and Trono 2003

Lentiviral Vectors

A retroviral vector siRNA delivery system has been developed based on a commercial vector utilizing the murine stem cell virus (MSCV). An shRNA was expressed using a U6 promoter and stable suppression of the human target gene was achieved. Retroviral vectors could have great utility for the transfection of primary cells that were previously refractory to siRNA or plasmid DNA transfection. This has in fact been observed in a number of cases with lentiviral vectors. Lentiviruses are a subclass of retrovirus derived from HIV that infects non-replicating cells, allows longer-term protein production and is not subject to silencing imposed on other retroviral vectors. A lentiviral vector with a U6 promoter was shown to express shRNA in a transduced population of primary T cells. Lentiviral vectors have also been shown to be effective for siRNA delivery in primary dendritic cells and non-cycling mammalian cells, stem cells, zygotes, and their differentiated progeny.

A number of different inducible vectors have been developed which allow turning on and off the synthesis of siRNAs in human and murine cells. The induction of synthesis can be accomplished with tetracycline, the analog doxycycline, or ecdysone.

Transfection

For some cell lines, transfection or electroporation may be utilized to achieve stable knockdowns with siRNAs. Transfection does not entail the time required for construction of viral vectors. Many transfection reagents combine two components, a novel cationic lipid formulation for siRNA binding and with a neutral lipid, such as dioleoylphosphatidyl-ethanolamine (DOPE), to allow escape from the endosome when the complex is within the cell. Other transfection reagents combine a cellular protein with a polyamine. Some cell lines are not susceptible to transfection of plasmid DNA.

Companies like Invitrogen (Carlsbad, CA) offer charged and neutral lipids, such as the effective Lipofectamine™2000 transfection reagent.

In vivo siRNA Delivery

Recently, progress has been seen in the delivery of siRNAs *in vivo*. For example, in two studies, siRNAs were injected under hydrostatic pressure into the mouse tail vein, and silencing of reporter transgenes was observed in different tissues. Another method of effective *in vivo* introduction is the direct injection of synthetic modified RNAi into tumors. To improve the delivery of siRNAs, the molecules are coated with protective lipid molecules, such as liposomes, and by attaching ligands that target specific cells to increase the specificity of delivery. Chemically modified synthetics may offer better stability and longer duration of effectiveness than standard siRNA for *in vivo* applications. Researchers are also turning to viral delivery, already proven for *in vivo* delivery of expressed genes, as an effective means of generating RNAi *in vivo*.

Peptide-based Approaches

New delivery systems include peptide-based approaches. Peptide-mediated delivery has been suggested as a modification to current methodologies that would allow more efficient delivery of siRNAs into cells. Cationic peptides that traverse plasma membranes of many mammalian cells are termed "protein transduction domains" (PTD) or "cell penetrating peptides." PTDs that have been used include TAT, Antennapedia (Antp) and poly-arginine peptide. Multifunctional peptides, cationic amphipathic peptides containing a number of histidines, have also been shown to bind DNA and destabilize membranes thereby allowing nucleic acid transfer.

Cholesterol Conjugation for Delivery

siRNAs conjugated to cholesterol have been shown to resulted in silencing of the endogenous apolipoprotein B (apoB) in mice *in vivo*.

Current areas of research in RNAi involve more efficient and effective delivery and maintaining activity for a longer time. As the first successful application of RNAi in mammalian cells was only in 2001 these are still early days for this technology. There has been a very rapid adoption of RNAi in many laboratories working on many different research problems. A number of different Web sites have rapid updates for staying abreast of the most recent RNAi research as shown in Table 3.

Table 3. Web Sources for Updated RNAi Research Information

Web site	Title	Location
PubMed	RNAi interference search	www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Search&db=PubMed&term=RNAi%20interference&dispmax=200
PubMed	siRNA search	www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Search&db=PubMed&term=siRNA&dispmax=200
BioS	RNAi sequences used	www.jbios.co.jp/RNAisequence.htm

Further Readings

For further reading, a number of recent reviews describing various aspects of RNAi is provided below.

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RNAi Applications in Mammalian Cells

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RNA interference, or RNAi, is a gene silencing mechanism originally elucidated in plants, *Caenorhabditis elegans*, and *Drosophila* (1,2). Mechanistically, RNAi results in sequence-specific destruction of mRNAs, allowing targeted knockdown of gene expression. Subsequent to the discovery of RNAi in plants and lower eukaryotes, it was demonstrated that RNAi occurs in mammalian cells (3,4), a finding that has prompted considerable interest in RNAi as a genetic tool and therapeutic agent. Gene silencing via the RNAi pathway appears to be activated by a double-stranded RNA (dsRNA) "trigger," which is processed by a cellular RNase III family enzyme called Dicer into short 21–25 nucleotide dsRNAs referred to as small interfering RNAs (siRNAs). The siRNAs are unwound, and one of the two strands becomes incorporated into a multiprotein complex that has been termed the RNA-induced silencing complex or RISC (5). The strand that enters RISC from the siRNA duplex appears to be the one with the less thermodynamically stable 5′ end (6,7). Once in RISC, the siRNA strand hybridizes with complementary target mRNA(s), activating an endonuclease within RISC that cleaves the target, resulting in its destruction. RISC can subsequently be recycled and can degrade multiple target mRNAs. This article will give an overview of the methods and some examples of RNAi utilization in mammalian cells.

siRNA Design: General Parameters

Proper design is a critical consideration for siRNA-mediated silencing. Two basic siRNA schemes have been successfully employed. The first strategy uses two 21 nucleotide RNAs, typically with a two-nucleotide overhang at each 3′ end, that mimic the natural product of Dicer cleavage (Figure 1). The second approach, referred to as short hairpin RNAs, or shRNAs (Figure 1), uses sense and antisense sequences connected by a hairpin loop. RNA duplexes slightly longer than 21 nucleotides in length can also be used as these may be further processed intracellularly into 21–25 base siRNAs. Both siRNAs and shRNAs can be chemically synthesized and introduced into cells or expressed endogenously from a promoter. For endogenous expression, RNA polymerase III (Pol III) promoters have been utilized for transcribing si- and shRNAs, since they can be engineered to initiate transcription with the first base of the si/shRNA and because Pol III terminates transcription within a short run of 4–6 uridines (8,9). RNA polymerase II promoters have also been successfully used (10).

RNAi has also been elicited by transfecting cells with pools of siRNAs generated by cleavage of in vitro transcribed long dsRNAs with recombinant Dicer or RNase III (11,12) or with *Escherichia coli* RNase III (13). Pooled siRNAs usually result in potent knockdown of target mRNA(s) while allowing discrimination between homologous sequences in gene families or alternatively spliced mRNAs.

The choice of exogenously created siRNA or vector-delivered siRNA expression units will ultimately depend upon the application. Transfection of synthetic siRNAs provides only transient RNAi, but gives dose-response relationships that are easily controlled, making it more suitable for large-scale

applications and multiplexing. On the other hand, stable, endogenous expression of si/shRNAs from integrating or episomal replicating vectors can cause long-term inhibition of gene expression—an important consideration for some therapeutic applications, such as the treatment of chronic viral infections.

Selectivity of RNAi

Some examples of siRNAs that are highly sequence-specific can be found in studies using RNAi in cancers that are associated with novel fusion transcripts. In chronic myeloid leukemia (CML), fusion *bcr/abl* transcripts are present in nearly all CML patients, as well as in 30% of adults with acute lymphoblastic leukemia (ALL). Transiently transfected siRNAs directed against the region spanning the *bcr/abl* fusion joint specifically reduced levels of the fusion *bcr/abl* transcripts and of the encoded p210 protein, without affecting the endogenous *abl* and *bcr* messages, despite the fact that parts of the siRNAs share perfect complementarity with these nonfusion transcripts (14,15). Importantly, the siRNA-induced reduction of the oncogenic transcript resulted in a desired physiological effect of cells becoming more susceptible to apoptosis. High selectivity will be required in therapeutic applications for which delivery to non-leukemic cells cannot be controlled.

A cautionary note about selectivity comes from a study of Pol III expressed siRNAs targeting the fusion transcript characteristic of Ewing's sarcoma (16). In this disease, the translocation t(11;22)(q24;q12) produces an oncogenic EWS/Fli-1 fusion protein detected in 85% of Ewing's sarcoma and primitive neuroectodermal tumor cells. Two different expressed siRNAs targeting sequences specific to the fusion were utilized. Both EWS/Fli-1 siRNAS specifically reduced the fusion mRNA relative to cellular b-actin. However, one of the siRNAs containing 17 bases of homology to Fli-1 partially reduced endogenous Fli-1 mRNA and also resulted in unanticipated N-myc down-regulation, highlighting the importance of monitoring cross-suppression of endogenous transcripts when using RNAi.

Off-Target Effects

There are conflicting reports about the potential "off-targeting" of siRNAs in mammalian cells. One study of siRNAs targeting a reporter construct (used in concert with DNA array analyses) showed no significant changes in the expression levels of nontargeted endogenous cellular genes (17). In contrast, other investigations have demonstrated numerous off-target effects (18–20). In one case, the off-target effects were concentration-dependent, with higher concentrations of siRNAs generating more off-targeting (20). It is therefore important to identify siRNA targets that produce desired effects at minimal (nanomolar) concentrations to minimize unintended effects and to design appropriate controls in siRNA knockdown experiments.

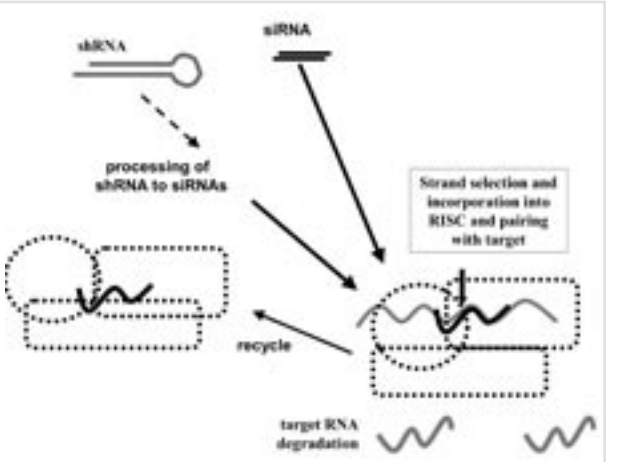

Figure 1. Mechanism of siRNA-directed degradation of target mRNA in mammalian cells. shRNAs are processed to siRNAs, which have a two base 3′ overhang and recessed 5′ phosphate. The antisense strand of the siRNA (relative to the target) is incorporated into the RNA induced silencing complex (RISC). RISC is comprised of several proteins, at least one of which is an Argonaut family member in addition to an RNase. The siRNA directs targeted cleavage of the mRNA and the RISC complex can recycle. siRNA, small interfering RNA; shRNA, short hairpin RNA.

Target Sequence Selection

An siRNA's ability to stimulate degradation of its target RNA is heavily dependent on target sequence selection (3,7,12,13,21,22), but the reasons are not fully understood. Base composition (GC content)

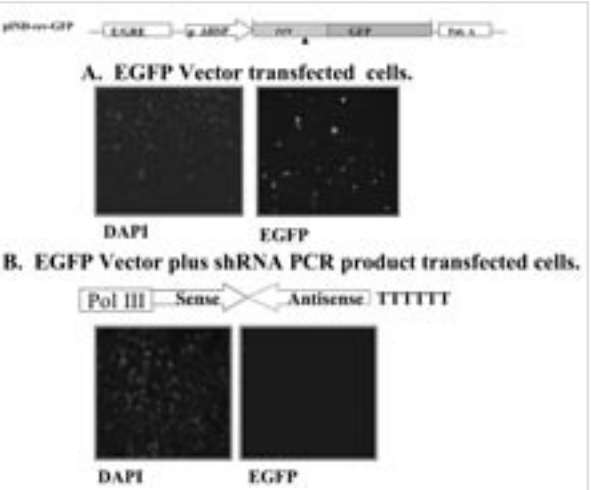

Figure 2. Example of a target fused to a reporter gene. The HIV-1 Rev cDNA has been joined to the EGFP to monitor RNAi function of anti-Rev shRNAs. Depicted at the top of the figure is an inducible system in which the Ecdysone response elements are fused to a basal promoter, and the system is induced with the Ecdysone analogue Ponasterone A (7,24). The target site for the anti-Rev shRNA expressed in panel B is depicted by a small vertical arrow. (A) HEK 293 cells transfected with the inducible Rev-EGFP expression vector alone. (B) HEK 293 cells transfected with Rev-EGFP fusion vector plus an anti-Rev encoding shRNA PCR product. A Pol III shRNA expression cassette is depicted. This was produced by PCR amplification as described in the text. The sense, antisense, and terminator region (Ts) are depicted. (A and B) The cell nuclei are stained with DAPI in the left-hand panels, and the right-hand panels depict EGFP fluorescence. The anti-Rev shRNA encoded by the transfected PCR product has down-regulated EGFP expression in the right-hand panel of B. HIV-1, human immunodeficiency virus type 1; EGFP, enhanced green fluorescent protein; RNAi, RNA interference; shRNA, short hairpin RNA; Pol III, RNA polymerase III.

alone does not seem to be a factor (12). More subtle structural aspects of the siRNA itself and of the target sequence, such as the ability to adopt the A-form helical structure (23) common to dsRNAs, may affect the efficiency of target site cleavage within the RISC complex. Studies utilizing methods to determine optimal in vivo ribozyme or antisense DNA target sites may be instructive. General RNA folding prediction software, such as mfold, do not reliably predict useful siRNA target sequences (see http://www.bioinfo.rpi.edu/~zukerm/seqanal/), but several commercial and academic web sites have useful algorithms for choosing siRNA target sequences. None of the in silico methods above takes into consideration the role of RNA chaperone proteins on inter- and intramolecular RNA interactions. A cell extract method that scans transcripts with inexpensive DNA oligonucleotides has proven to be useful for identifying ribozyme, antisense, and RNAi-susceptible target sequences (4,18–20).

Another technique for identifying effective RNAi target sequences is to use a construct that includes the target gene of interest fused to a reporter, such as the enhanced green fluorescent protein (EGFP) (24) (Figure 2). The siRNA-directed cleavage of the target gene sequence will result in loss of EGFP fluorescence, which can be quantitated. This approach has several advantages: (*i*) in vivo application, (*ii*) rapid readout, and (*iii*) effectiveness in standard tissue culture.

In an effort to rapidly and efficiently identify RNAi sensitive sequences in cells, we developed a PCR-based procedure for generating Pol III shRNA transcription units. The in vitro-amplified PCR products are transfected into mammalian cells containing the target mRNA (24). A number of transcription units expressing different si/shRNAs targeting different sequences in the gene of interest can be rapidly synthesized and tested, since the transcriptional units are tagged with a 3′ sequence that allows easy PCR amplification from transfected cells, enabling researchers to test multiple shRNA sequences and isolate the most potent ones following cell sorting (24).

Targeting Human Immunodeficiency Virus with RNAi

Human immunodeficiency virus (HIV) has become a prime target for RNAi due to problems with resistance to current drugs and the high cost of new drug development (25,26). Synthetic and expressed siRNAs have been used to target a number of early and late viral mRNAs as well as cellular cofactors (7,27,28). Successful inhibition of HIV replication has been achieved in numerous human cell lines and primary cells including hematopoietic stem cell-derived T lymphocytes and macrophages (29).

The use of siRNA as a potential therapeutic agent in the treatment of HIV and hepatitis C virus (HCV) (30–33) is complicated by the high mutation rates of these viruses during replication. Since even one nucleotide mismatch between an siRNA and its target can drastically lower activity (6,34), the appearance of escape mutants is likely. Selecting targets in highly conserved sequences (where mutations are presumably deleterious to the virus) and using combinations of si/shRNAs targeting different sites within the HIV viral genome will reduce the likelihood of resistant variants arising. The multitargeting approaches of RNAi make this method a potentially powerful therapeutic for the treatment of viral infection.

Conclusion

RNAi has rapidly gained in popularity as the method of choice for target-specific down-regulation of gene expression in eukaryotic systems. Because unwanted side effects can occur, careful attention to controls must be utilized in RNAi-mediated knockdowns. As we learn more about the mechanisms involved in this phenomenon, it may be possible to increase specificity and avoid off-targeting problems by proper design of si/shRNAs. It is clear that use of this powerful biological phenomenon will continue to expand and provide fascinating new insights into gene regulation as well as exciting therapeutic applications.

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siRNA Cell Arrays for High-Content Screening Microscopy

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Abstract

RNA interference (RNAi) is a recent advance that provides the possibility to reduce the expression of specific target genes in cultured mammalian cells with potential applications on a genome-wide scale. However, to achieve this, robust methodologies that allow automated and efficient delivery of small interfering RNAs (siRNAs) into living cultured cells and reliable quality control of siRNA function must be in place. Here we describe the production of cell arrays for reverse transfection of tissue culture cells with siRNA and plasmid DNA suitable for subsequent high-content screening microscopy applications. All the necessary transfection components are mixed prior to the robotic spotting on noncoated chambered coverglass tissue culture dishes, which are ideally suited for time-lapse microscopy applications in living cells. The addition of fibronectin to the spotting solution improves cell adherence. After cell seeding, no further cell culture manipulations, such as medium changes or the addition of 7serum, are needed. Adaptation of the cell density improves autofocus performance for high-quality data acquisition and cell recognition. The co-transfection of a nonspecific fluorescently labeled DNA oligomer with the specific siRNA helps to mark each successfully transfected cell and cell cluster. We demonstrate such an siRNA cell array in a microscope-based functional assay in living cells to determine the effect of various siRNA oligonucleotides against endogenous targets on cellular secretion.

INTRODUCTION

The completion of sequencing the genomes of a number of organisms now provides us with the opportunity to understand how these lists of genes give rise to cellular behavior governed through protein interaction networks. Within this framework, one goal of proteomics is to classify proteins according to their function. In order to do this, robust protocols need to be available that can be applied to large sets of molecules. Recently, Ziauddin and colleagues (1) have developed such a tool for the proteomics field, namely, a microarray of printed plasmid DNAs for reverse transfection of living cells. Such microarrays are prepared by first printing a coated glass slide with an array of plasmid DNAs. Transfection reagents are then overlaid and, finally, cells are plated onto these slides, resulting in clusters of cells that, with the help of the transfection reagent (reverse transfection), express the respective cDNAs at each location. While cDNA overexpression has certainly enhanced our understanding of gene function, the ability to selectively down-regulate genes is now proving even more powerful. In particular, RNA interference (RNAi) utilizing small interfering RNAs (siRNAs) is a recent advance that provides the possibility of reducing gene expression at the post-transcriptional level in cultured mammalian cells (2). This technique is one valuable step toward determining gene function and can be theoretically applied on a genome-wide scale (3).

Here we describe the development, testing, and application of a protocol for siRNA microarray production that allows spotting of the siRNAs and transfection reagents in only two steps. The procedure is sufficiently robust to be applied through automation on a large scale to the down-regulation of a variety of endogenous proteins. Furthermore, the quality of data obtained from this method is ideally suited for microscope-based functional assays designed to determine gene function in living cells.

MATERIALS AND METHODS

RNAi Oligonucleotides

Oligonucleotides (Qiagen, Valencia, CA, USA) used for RNAi were as follows. For human β-COP (GenBank® accession no. NM_016451), the target sequences were 161–181 nucleotides (5′-AACUUCCUGGACUUCUGAUGA-3′) and 1566–1586 nucleotides (5′′-AACUCAGAGUGCCCUUAGCAG-3′) downstream from the ATG. For human GM130 (accession no. AF248953; BD Biosciences, San Jose, CA, USA), the target sequence was 543–563 nucleotides (5′-AAUUACGGAUCAGUUGGAAGA-3′), and for human Sec31 (accession no. AF139184), 529–549 nucleotides (5′-AACAGACAAGUUCAGCAUAUU-3′) downstream from the ATG. The target sequence of the control oligonucleotide was 5′-AAUUCUCCGAACGUGUCACGU-3′. As a control, a nonsilencing rhodamine-labeled siRNA (si control; si, small interfering) with the sequence 5′-UUCUCCGAACGUGUCACG-3′ was used.

In each case, pairs of complementary RNAs with 3′ dT overhangs were synthesized, annealed, and purified. Lyophilized oligonucleotide duplexes were resuspended in the supplied resuspension buffer (Qiagen) at 20 µm. The Cy™3-labeled DNA marker oligomer with the sequence 5′-TGACGTTCTATAGCGACGGCCAGT-3′ was obtained from BioSpring (Frankfurt, Germany). The labeling of oligomers with rhodamine or Cy3 was always at the 5′ end.

Preparation of Cell Arrays

The siRNA-gelatin transfection solution was prepared in 384-well plates (Nalge Nunc International, Rochester, NY, USA) as follows. Plasmid (500 ng) and/or 1 µL of siRNA solution (20 µm in RNA dilution buffer; Qiagen), and 7.5 µL EC buffer (Qiagen) containing 0.2 M sucrose were incubated for 10 min at room temperature, mixed with 4.5 µL Effectene® (Qiagen), and incubated for an additional 10 min at room temperature with 7.25 µL of 0.08% gelatin (Sigma-Aldrich, St. Louis, MO, USA) and 3.5×10^{-4}% fibronectin **(**Sigma-Aldrich).

For transfections with Oligofectamine™ (Invitrogen, La Jolla, CA, USA), 1 µL siRNA solution was added to 8.5 µL OptiMEM™ (Invitrogen) and incubated for 10 min. OptiMEM (1.6 µL) and 0.4 µL Oligofectamine were mixed and incubated for 10 min. These two separate solutions were subsequently mixed and incubated for another 10 min. Then, 8.5 µL of 0.2% gelatin and 30 µL of phosphate-buffered saline (PBS) were added.

In those experiments where a Cy3-labeled DNA oligonucleotide was used as transfection marker, 0.5 µL of a 40-µm marker solution was added together with the siRNA or DNA to be transfected. The solutions were arrayed onto single-well, chambered Lab-Tek™ coverglass tissue culture dishes (Nalge Nunc International) using a ChipWriter™ Compact Robot (Bio-Rad Laboratories, Hercules, CA, USA). Solid pins (Eurogentec, Seraing, Belgium) and SMP10™ quill pins (TeleChem International), resulting in a spot diameter of approximately 400 µm for all experiments, were used. The spot volume was approximately 4 nL. After printing, 1.25×10^5 HeLa, MCF7, COS7L, or HEK293 cells were plated on the Lab-Tek slide in a total volume of 2.5 µL culture medium [Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS), 2 µm glutamine, 100 U/µL penicillin, and 100 mg/µL streptomycin] and incubated for 44 h at 37°C and 5% CO_2.

Immunostaining of Cells and Transport Assay

For immunostaining, the cells were first fixed in -20°C methanol for 4 min, followed by 2 washes with PBS. The monoclonal antibody against β-COP (mAD; Reference 4) was applied for 10 min, followed by washing of the cells and incubation with anti-mouse Alexa488- (Molecular Probes, Eugene, OR, USA) or Cy5- (Amersham Biosciences, Piscataway, NJ, USA) conjugated secondary antibodies. Finally,

Hoechst 33342 (1 mg/µL final concentration; Sigma-Aldrich) was added for 10 min to stain the cell nuclei. For the transport assay, cell arrays were prepared as above and then incubated for 26 h prior to infection with recombinant adenoviruses expressing cyan fluorescent protein (CFP)-tagged ts-O45-G for 1 h, followed by incubation at the restrictive temperature of 39.5°C for 16 h (5). Ts-O45-G was released from the endoplasmic reticulum (ER) at 32°C for 1 h in the presence of 100 mg/µL cycloheximide (Calbiochem, San Diego, CA, USA). The cells were fixed with 3.5% paraformaldehyde for 20 min prior to staining with antibodies against an extracellular epitope of the ts-O45-G protein (4). Secondary antibodies were prepared as described above.

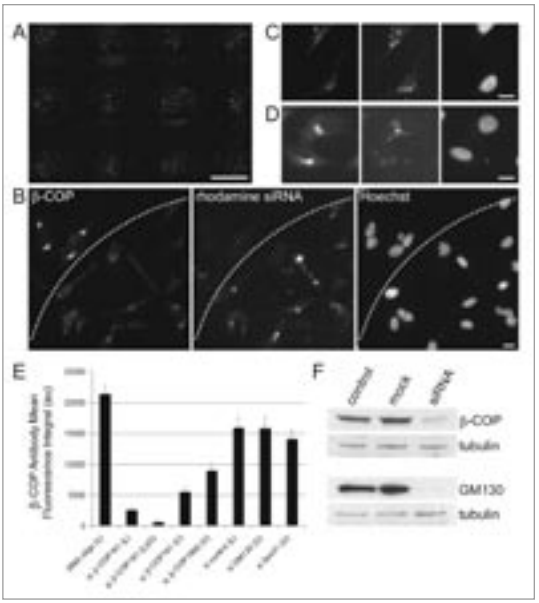

Figure 1. Specific down-regulation of endogenous β-COP on a small interfering RNA (siRNA) cell array in HeLa cells. (A) Example of a 4 × 3 array of rhodamine-labeled siRNA oligonucleotides against β-COP, and (B) their effect on the down-regulation of β-COP protein within the limit of the spotted area, as indicated by the dotted line. (C) Further enlargement of the cells within the area of this spot shows lack of β-COP staining in those cells containing the siRNA molecules, (D) whereas within the area of a rhodamine-labeled control (nonsilencing) siRNA spot, normal β-COP staining in those cells containing the siRNA molecules is observed. Bars indicate 500 mm in panel A and 10 mm in panels B–D. (E) A quantification of the β-COP immunostaining in spots containing siRNA oligonucleotides against various proteins of the secretory pathway. L denotes that the oligonucleotides were labeled, U denotes that they were unlabeled but co-transfected with the Cy3 marker DNA oligonucleotide, and G denotes that the measurement was made from the global area of the spot. (F) The efficacy of the siRNAs against β-COP and GM130 was also confirmed by Western blot analysis. Blots for tubulin from the same cells are shown to indicate protein loadings. Oligo, oligonucleotide; si, small interfering; au, arbitrary units.

Data Acquisition and Analysis

Images were acquired with Axio Vision™ 3.1 on an Axiovert™ 200M Microscope (Carl Zeiss, Rochester, NY, USA) equipped with a 20×/0.75 air Fluar® objective (Carl Zeiss) and an AxioCam HR™ CCD camera (Carl Zeiss). The filters used were standard filters (no. 34 for Hoechst, no. 6 for CFP, no. 44 for Alexa 488, no. 43 for Cy3 and rhodamine, and no. 26 for Cy5). Exposure times varied from 57 ms for Hoechst, 800 ms for CFP, 300 ms for GFP, and 500 ms for Cy3 and rhodamine to 6500 ms for Cy5 and were kept constant for all experiments.

The images were background-corrected by subtracting the average pixel value in a blank region of the image. Borders of the spot area could be estimated by Cy3 or rhodamine labeling of cells (Figure 1A). Transfection efficiencies were then determined by visual inspection and dividing the number of successfully transfected cells (indicated by their oligomer-specific Cy3 or rhodamine labeling (Figure 1) by the total number of cells in a spot area.

Quantification of single-cell fluorescence was performed with MetaMorph® (Universal Imaging, Downingtown, PA, USA). After background subtraction, a region around each cell of interest was defined manually (radius: 50 image pixels). In each region, the average intensity was measured, and the mean values with the standard deviation of the means for all regions were calculated. Intensities are expressed in arbitrary units.

The ts-O45-G transport efficiency was calculated by dividing for each cell the intensity obtained for the ts-O45-G at the cell surface (Cy5) by the intensity of the CFP-ts-O45-G within the cell. The average

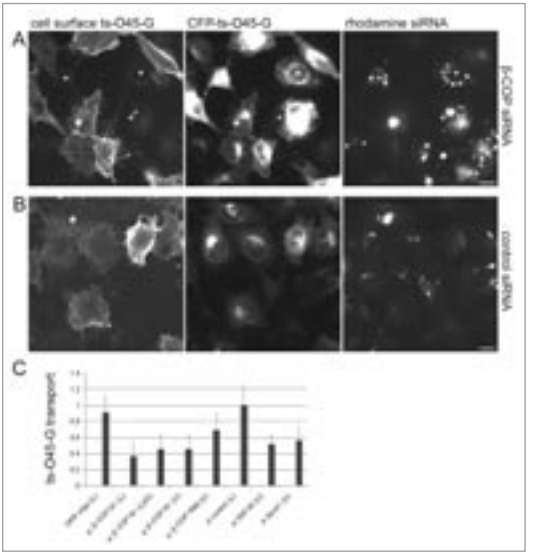

Figure 2. Use of a small interfering RNA (siRNA) cell array in an assay to measure ts-O45-G transport to the plasma membrane in HeLa cells. (A) Images from within a spot of transfected siRNA against β-COP showing the presence of the secretory marker ts-O45-G on the cell surface, the total CFP-ts-O45-G in the cells, and the presence of the labeled siRNAs. Asterisks mark those cells showing the presence of siRNAs, but no transport of ts-O45-G to the cell surface. (B) Similar experiment as shown in panel A but using a control siRNA. Bars indicate 10 µm. (C) Quantification of ts-O45-G transport to the cell surface in spots containing siRNA oligonucleotides against various proteins of the secretory pathway. L denotes that the oligonucleotides were labeled, U denotes they were unlabeled but co-transfected with the Cy3 marker DNA oligonucleotide, and G denotes that the measurement was made from the global area of the spot. Oligo, oligonucleotide; CFP, cyan fluorescent protein; si, small interfering.

values obtained were then normalized to the si control (1.0) spotted on the same slide.

Western Blot Analysis

HeLa cells were cultured in 6-well plates prior to transfection with siRNA oligonucleotides against β-COP or GM130. For transfections with Oligofectamine, 10 µL siRNA solution were added to 170 µL OptiMEM and incubated for 10 min. Sixteen microliters of OptiMEM and 4 µL Oligofectamine were mixed and incubated for 10 min. These two separate solutions were subsequently mixed and incubated for another 20 min. The cells were washed once with serum-free medium and then overlaid with 800 µL of the same medium. The transfection mixture was added drop-wise to the cells, and then incubated for 4 h. Finally, the cells were overlaid with 500 µL of medium containing 30% serum (10% final concentration). After 44 h, the cells were lysed in radioimmunoprecipitation (RIPA) buffer [50 µm Tris, pH 7.2, 150 µm NaCl, 1% Triton® X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)], and the total protein concentrations were determined. Twelve micrograms of each extract were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels followed by transfer to polyvinylidene difluoride (PVDF) membranes. Membranes were probed with antibodies against β-COP, GM130, or α-tubulin (Neomarkers, Fremont, CA, USA), followed by appropriate secondary antibodies and electrochemiluminescence (ECL) detection.

RESULTS AND DISCUSSION

In order to be able to apply reverse transfection to experiments in living cells, we decided to use chambered coverglass tissue culture dishes for spotting the cDNA and siRNA molecules. These have the advantage that they easily permit the cellular medium to be changed, facilitate automation of immunostaining protocols and, most importantly, allow high-resolution functional assays in living cells on any inverted microscope.

For array production, we developed a two-step protocol in which, in the first step, the transfection reagent used is directly included with a gelatin/siRNA (DNA)/fibronectin mixture for the spotting on uncoated chambered coverglass tissue culture dishes.

The optimal range of fibronectin concentration was determined to be between 3.5×10^{-4}% and 5.25×10^{-4}%. In this range, the transfection efficiency increased 1.5-fold, and cell adherence 2-fold per spot, compared to the same solution without fibronectin. We used 3.5×10^{-4}% fibronectin for further experiments.

For a spot diameter of 400 µm and a spot-to-spot distance of 1125 µm, one can apply 384 samples per Lab-Tek chamber. Decreasing the spot-to-spot distance to 642 µm allows up to 1176 spots to be printed on a single Lab-Tek slide, thereby accommodating samples from more than three 384-well plates.

Reverse transfection by manual spotting, for example, into 8-well Lab-Tek chambers is also possible. Usually, we perform this by placing a mask underneath the Lab-Tek chamber to facilitate a regular spot-to-spot spacing. Typical spot volumes are 0.2 µL, and about 9 spots can be printed into one of the 8-well chambers.

This all-in-one mixture thereby expedites sample preparation time and provides the reagents necessary for cell adherence on uncoated glass surfaces. In the second step, cells are plated onto these arrays in complete growth medium without further changes before data acquisition and analyses. In addition, we found that this transfection mixture is highly suited to a lower density of cells than previously used, ultimately allowing more detailed image analysis. Reducing in this way the reverse transfection protocol to two steps allows automation of the entire procedure as required for large-scale projects and is different from earlier protocols (6,7) that involved medium changes or the addition of serum. One problem when preparing arrays of siRNAs targeting endogenous genes is the subsequent location of the exact site of the spotted material. To address this, we either used directly labeled siRNAs (Figures 1–3) or, to make such down-regulation cell arrays more universal and less expensive, unlabeled siRNAs were mixed with a nonrelevant Cy3-labeled DNA oligonucleotide serving as the transfection marker.

In the first phase, we compared HeLa, MCF7, COS7L, and HEK293 cells for their transfection efficiencies, growth behavior, and morphology. The highest transfection efficiencies (>95%) were achieved with HEK293 cells. However, these cells have the disadvantage that they tend to grow on top of each other, making automated image acquisition and analysis and, in particular, focus identification and cell segmentation, difficult. In addition, of all the cell types tested, they were the least adherent, and 24 h after plating cells onto spotted siRNAs, they had already detached. The lowest transfection efficiencies (70%) were achieved with COS7L cells. With MCF7 and HeLa cells, comparable transfection efficiencies of 80%–90% were obtained, and even 50 h after plating, HeLa cells were still highly adherent in the spot areas. For subsequent experiments, HeLa cells were used.

As a first test of our modified protocols in applications to down-regulate endogenous target genes, we designed a number of siRNA oligonucleotides against known proteins of the secretory pathway, including the β subunit of the coat protein complex COPI, an essential proteinaceous coat required for membrane transport through and the integrity of the Golgi complex (8). Cell arrays produced with these siRNAs were incubated for 44 h before fixing and staining with a monoclonal anti-β-COP antibody to monitor down-regulation of endogenous β-COP in individual cells. In the cell cluster where the β-COP-specific siRNA (siβ-COP_{161}) could be visualized in the rhodamine (marker) channel, almost no immunostaining of the β-COP protein in comparison to nontransfected cells outside the spot cluster could be observed, indicating effective down-regulation of the target protein by the labeled siRNA (Figure 1, A–C). In clusters where a rhodamine-labeled control siRNA known to have no target in mammalian cells was transfected, cells appeared to have normal β-COP staining, which was indistinguishable from those cells

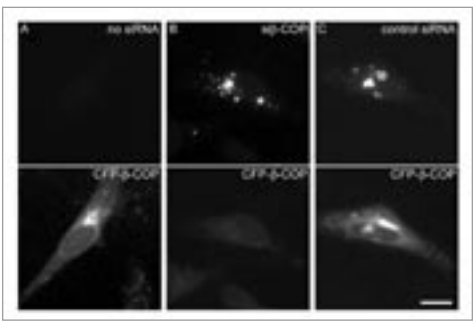

Figure 3. Co-transfection on arrays spotted with cDNA and small interfering RNA (siRNA) molecules in HeLa cells. (A) Example of a CFP-β-COP-expressing cell in a spot transfected with the respective plasmid expression vector only. (B) Cell showing co-transfection of CFP-β-COP and a corresponding siRNA, resulting in the lack of cDNA expression. No cells expressing CFP-β-COP could be detected in these spots. (C) Cell showing co-transfection of CFP-β-COP and a control siRNA. Bar indicates 10 µm. CFP, cyan fluorescent protein.

outside the spot area (Figure 1D). Quantification demonstrated that in β-COP-specific siRNA-transfected cells, only about 15% of β-COP-specific staining remained compared to control transfected cells (Figure 1E). When an identical but unlabeled siRNA against β-COP was co-transfected with the Cy3-labeled marker DNA oligonucleotide, endogenous β-COP was also efficiently suppressed (Figure 1E), suggesting that the addition of the labeled DNA oligonucleotide as a marker for the spot and transfected cells has only little effect on the uptake and efficacy of the target-specific siRNA molecules. We performed a control experiment in parallel, using "conventional" transfection of HeLa cells with the siβ-COP_{161} oligonucleotides to determine the extent of down-regulation as determined by Western blot analysis. As shown in Figure 1F, the levels of β-COP protein were reduced to similar levels as that observed by the analysis of the cell array, while tubulin was unaffected. Together, this indicates that, with respect to gene down-regulation, the cell array approach gives comparable results to biochemical techniques.

We also observed that within the global boundary of the spot, a number of the cells had no detectable rhodamine fluorescence, although the β-COP staining was clearly reduced. While measuring these cells, we observed that the average total intensities of the β-COP staining was in fact even lower than in those cells where the rhodamine-labeled siRNA molecules were still visible (Figure 1E). Because it was therefore clear that the down-regulation was also effective in all the cells within the global area of the spot, for subsequent evaluation we took all the cells within this area into account. One likely explanation of this visual disparity is that the rhodamine label of the siRNA is partially degraded during siRNA processing in the cell. To further test the quality of the quantitative analysis from such cell arrays, we designed a second siRNA against β-COP (siβ-COP_{1566}), which also caused a visible reduction in the level of β-COP protein; however, the quantification confirmed that this was not as effective as the first oligonucleotide siβ-COP_{161} (Figure 1E). Therefore, such siRNA cell arrays also appear to be a useful means to compare the efficacy of multiple siRNA molecules (7). When siRNAs against Sec31, a component of the COPII complex (9) and against the Golgi matrix protein GM130 (10) were transfected, little effect on the strength of the β-COP staining was observed compared to control transfected cells, further highlighting the specificity of the approach (Figure 1E). The down-regulation of GM130 and Sec31 was however effective in these cells when checked by Western blot analysis (Figure 1F; data not shown).

We next applied the siRNA cell array to a functional assay in living cells. In particular, we were interested in screening for the effect of down-regulating individual components of the secretory pathway on the secretion of a specific transport marker. To perform this assay, we made use of recombinant adenoviruses encoding a fluorescently tagged temperature-sensitive variant of the viral glycoprotein VSV-G (ts-O45-G; References 4 and 11). This marker is particularly well suited to such assays because, at its restrictive temperature, it remains misfolded in the ER, but on lowering the temperature, it is rapidly transported to the cell surface. Visual analysis of the arrays revealed that in the spots containing siRNA against β-COP, the transport of the ts-O45-G to the cell surface was severely reduced in many of the cells (Figure 2A). In contrast, within the spot containing the control siRNA, the uptake did not apparently influence the transport (Figure 2B). For each spot, the transport efficiency was determined and thereby a measurement of the effectiveness of the siRNA in this functional assay. The control siRNA and DNA oligonucleotides alone had no significant effect on ts-O45-G secretion, whereas in the cells depleted of β-COP, transport was reduced to less than 40% of the control level. Furthermore, even in the spot containing the less effective siRNA against β-COP_{1566}, the transport efficiency was also reduced. These data are in good agreement with the role of COPI in retrograde transport from post-ER membranes (13, and references therein), and a reduction of cellular β-COP levels down to 15% of control values should clearly affect ts-O45-G transport as observed here. However, as β-COP is only indirectly involved in anterograde transport, the amounts of β-COP in siRNA-treated cells may still be enough to allow some ts-O45-G to be transported and provides a possible explanation for still 40% of ts-O45-G arriving at the plasma membrane in our experiments at strongly reduced β-COP levels.

Of particular interest, however, was that in the spots of siRNA against GM130 and Sec31, ts-O45-G transport was reduced to approximately 50% of control values (Figure 2C). Although these results are perhaps not surprising because these proteins are important components of the secretory pathway, they provide evidence that such siRNA cell arrays are sufficiently sensitive to be used in functional

assays such as this one to screen large panels of siRNA molecules designed against endogenous targets.

Finally, we tested our reverse transfection protocol for its ability to be able to deliver both DNA and siRNA molecules into the same cells. Such a possibility is important for large-scale screening projects in which the cDNA encoding a protein of unknown function is available, but an antibody is not. In such a case, it is imperative to know that an siRNA targeted against the protein of unknown function is effectively down-regulating the protein. To demonstrate this, we created an array with spots containing both a cDNA encoding CFP-β-COP and the corresponding siRNA (Figure 3). Between 20 and 40 CFP-β-COP-expressing cells were clearly visible in those spots transfecting either the cDNA alone (Figure 3A) or the cDNA with the control siRNA oligonucleotides (Figure 3C). In contrast, the expression of CFP-β-COP was almost not detectable when the siβ-COP_{161} oligonucleotides were co-transfected with the CFP-β-COP cDNA (Figure 3B).

Such an siRNA array should prove an ideal means to utilize the already available large resources of fluorescently tagged cDNAs of unknown function (see http://gfp-cdna.embl.de/; Reference 12) in combination with libraries of siRNA molecules, and thereby determine siRNA efficacy prior to their routine use. Together, our results show that the improved protocols we have developed for the creation of cell arrays are highly robust, equally applicable to cDNA overexpression and siRNA down-regulation, and are suitable for application in high-resolution functional assays in living cells to determine protein function.

Acknowledgments

We thank Brigitte Joggerst for technical assistance. This work has been supported by the BMBF with grants 01GR0101 and 01KW0013 and by a grant from the Verbundforschung Baden-Wuerttemberg (grant no. 24-720.431-1-2/2) to R.P.

Competing Interests Statement

The authors declare that they have no competing interests.

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Improved siRNA-mediated silencing in refractory adherent cell lines by detachment and transfection in suspension

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Cationic liposome-based agents (1) are commonly used for cellular delivery (transfection) of oligonucleotides such as short interfering RNA (siRNA) (2). When targeting endogenous gene expression, transfection efficiency is a critical parameter that may vary substantially depending on a combination of cell line and transfection agent, thereby necessitating a time-consuming and expensive optimization of transfection conditions (http://www.molecula.com/new/transfection_optimization.html). It has been previously reported that the human keratinocyte cell line HaCaT is effectively transfected with an siRNA of optimal activity (hTF167i), which consistently depleted the endogenous target mRNA expression by 90% (3), using the cationic liposome-based agent Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). This siRNA proved to be substantially less effective in the human melanoma cell line LOX (4) under a range of transfection conditions, using either Lipofectamine 2000 or the lipid formulation RNAiFect™ (Qiagen, Hilden, Germany). This suggested that the LOX cells are relatively refractory to transfection with standard methods. Instead of investigating other transfection agents in an attempt to find one that works well with this cell line, alternate methods of utilizing the available agents were considered.

Detachment and co-incubation of cells with complexes while in suspension was considered a possible way of improving transfection efficiency. For this modified transfection protocol, complexes were prepared as usual in 20% of the final transfection volume (3). Briefly, siRNA was diluted in serum-free medium (1/10 of the final transfection volume) and mixed with an equal volume of medium-diluted liposomes according to the manufacturer's instructions. Cells were prepared for transfection as follows. They were washed twice with phosphate-buffered saline (PBS) and detached by trypsinization. Trypsin was inactivated by the addition of a 1.5 mL serum/75 cm^2 tissue flask of cells. The cell/serum suspension was diluted 10-fold with PBS and subjected to centrifugation at 400–500× *g* for 5 min. The supernatant was removed, and the cells were resuspended in PBS. Following a new centrifugation step and removal of the supernatant, the cells were resuspended in serum-free medium and counted. The cell suspension was adjusted to the appropriate density (typically 0.8–1.0 × 10^6 cells/mL, see below) and mixed with complexes by careful pipeting before seeding into wells or dishes. Cells were then incubated for 5 h before replacement of medium. Twice the number of cells normally seeded for next-day transfection of adherent cells were used when performing transfections in suspension (typically 6–8 × 10^5 cells in 1.0 mL/36-mm well). This was done to compensate for cell expansion from time of seeding to transfection when transfecting adherent cells and for the projected loss of cells that had not attached properly at the time of medium replacement when performing transfections in suspension.

Transfection of LOX cells adherently with hTF167i siRNA under a range of different transfection conditions resulted in knockdown of endogenous target mRNA in the ranges of 51%–57% and 48%–57% (Figure 1, right panel) for the respective transfection agents Lipofectamine 2000 and RNAiFect.

However, when the cells were transfected in suspension, silencing of gene expression improved to 82% and 76% for Lipofectamine 2000 and RNAiFect, respectively (Figure 1, left panel). In a follow-up experiment, although overall levels of silencing were generally reduced, similar relative improvements in silencing were seen (data not shown). After these encouraging observations, the applicability of the above methodology to other combinations of siRNA and difficult-to-transfect cell lines was investigated. An siRNA against murine Tissue Factor (mTF), mTF223i, depletes expression of mTF in the murine B16 melanoma cell line by 80% (M. Amarzguioui, Q. Peng, T. Holen, V. Vasovic, E. Babaie, J.M. Nesland, and H. Prydz, unpublished data). In another melanoma cell line, K1735-M4 (5,6), no more than 50% knockdown was obtained under the same transfection conditions. Experiments were undertaken in which two different active mTF siRNA were evaluated for silencing efficiency when used to transfect K1735-M4 cells adherently or in suspension, with either Lipofectamine 2000 or RNAiFect. Transfection of cells in suspension substantially improved silencing for all combinations of siRNA and transfection agents (Figure 2). For mTF223i, transfection of adherent cells with Lipofectamine 2000 and RNAiFect resulted in (mean ± sd) 44% ± 9 ($n = 3$) and 59% ± 8% ($n = 3$) depletion of mTF mRNA, respectively. The corresponding knockdown levels for cells transfected in suspension with identically prepared complexes were (mean ± sd) 81% ± 3 ($n = 3$) and 84% ± 3 ($n = 3$). This represents a substantial improvement in the degree of silencing, which is likely to be of critical importance for many functional studies.

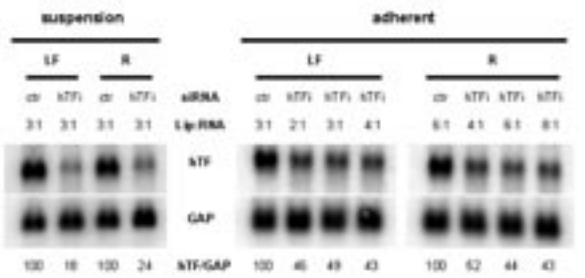

Figure 1. Northern blot analyses comparing short interfering RNA (siRNA)-mediated silencing of human Tissue Factor (hTF) mRNA expression in LOX cells under various transfection conditions. Data from a representative of two independent experiments are shown. Cells were transfected in 6-cm dishes either adherently (right panel) at 40%–50% confluency (cells plated at 7 × 105/dish 18 h before transfection) or in suspension (left panel) (1.5 × 106/dish) with 2.0 mL 100 nM siRNA complexed with either Lipofectamine 2000 (LF) or RNAiFect (R). ctr, mismatched control siRNA (mTF223i) targeting murine Tissue Factor (mTF); hTFi, active hTF-specific siRNA (hTF167i). Complexes had liposomes to RNA ratios (Lip:RNA, v/w, µL liposome to µg RNA) as indicated. Cells were harvested 48 h posttransfection for mRNA isolation using Dynabeads® Oligo(dT)25 (Dynal Biotech, Oslo, Norway). Northern blot analyses were hybridized with full-length cDNA probes for hTF and GAPDH in PerfectHyb™ Plus Hybridization Buffer (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. GAPDH expression served as loading and normalization control. Hybridized membranes were scanned, and images were analyzed using the Molecular Dynamics Storm® 860 scanner and ImageQuant™ 5.1 software (both from Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). GAP, GAPDH.

The silencing efficiencies achieved in more permissive (i.e., easier to transfect) cell lines by either the standard or the modified protocol, using the transfection agent Lipofectamine 2000, were subsequently compared. In MCF-7 cells, the level of silencing achieved by two highly effective siRNAs (wwox1 and wwox2) targeting WWOX mRNA was tested in parallel with both transfection protocols. In the first experiment, transfection in suspension improved the level of knockdown from 75% to 82% for wwox1 and from 71% to 80% for wwox2. Similar improvements were seen for both siRNA in a follow-up experiment (from 80% to 88% for wwox1 and from 85% to 89% for wwox2). Thus, although the differences were relatively small due to the high initial silencing efficiency, they were consistent across four separate side-by-side comparisons, with an average percentage point increase in silencing of 7.0% ± 2.2 ($n = 4$). The above data, in addition to preliminary data from two other human cell lines, suggest that performing transfections in suspension results in slightly improved results even for easily transfectable cell lines. Transfection of HaCaT cells in suspension did not result in significant improvement of silencing achieved with siRNA of low intrinsic activity. This further supports the notion that transfection in suspension increases the number of functionally transfected (i.e., silenced) cells, which in HaCaT cells is close to 100% even with the standard protocol.

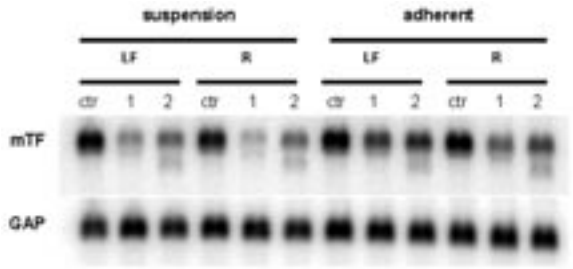

Figure 2. Northern blot analyses comparing short interfering RNA (siRNA)-mediated silencing of murine Tissue Factor (mTF) mRNA expression in K1735-M4 cells under various transfection conditions. Data from a representative of three independent experiments are shown. Ctr, mismatched control siRNA (hTF167i) targeting human Tissue Factor (hTF). Lanes 1 and 2, active mTF-specific siRNA (mTF223i and mTF321i, respectively). Complexes have optimized ratios (v/w) of liposomes to RNA of 3:1 for Lipofectamine 2000 (LF) and 6:1 for RNAiFect (R). Northern blot analyses were performed as described for Figure 1. GAP, GAPDH.

The reasons for the improvements in silencing observed with the modified protocol reported here are not clear. One possibility is that a more favorable cell surface environment for attachment of complexes is generated following partial removal of surface proteins by trypsinization. If this were the case, improvements should also be expected for cells normally cultured in suspension if they were to be subjected to a trypsinization step prior to mixing with the liposome complexes. However, Lipofectamine 2000-mediated transfection of Jurkat cells failed to demonstrate any effect of incorporating a 5-min trypsin treatment step in the preparation of cells for transfection (data not shown). Trypsinization may improve transfection efficiency of adherent cells through increased surface availability. It is also possible that the critical factor is the presence of complexes on the cells at the time when they undergo morphological changes associated with attachment to the surface of the culture plates or dishes, which may render them more easily transfectable.

In conclusion, this report has demonstrated that silencing of endogenous gene expression in cells that are normally resistant to transfection with two different commonly used agents can be improved substantially by mixing the complexes with cells in suspension and allowing the cells to attach in the presence of the complexes. This strategy has been shown to improve silencing for all combinations of refractory cell line, siRNA, and transfection agent tested. While the method has only been evaluated for the cationic liposome-based agent Lipofectamine 2000 and the lipid formulation RNAiFect, the applicability of the method is not expected to be restricted to the reagents mentioned above.

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Application of Stealth™ RNAi for directed differentiation of mouse embryonal carcinoma cells and embryonic stem cells

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Introduction

RNA interference (RNAi) describes the phenomenon by which double-stranded RNAs (dsRNAs) elicit degradation of a homologous target mRNA[1, 2]. In this pathway, the dsRNA duplex is believed to assemble with a series of proteins, referred to as the RNA-induced silencing complex (RISC), that guides hybridization of the siRNA antisense strand to its complementary target sequence and initiates cleavage of the target mRNA (reviewed by[3, 4]). Several years ago, the successful application of short dsRNAs, referred to as small interfering RNAs (siRNAs), for sequence-specific inhibition of target gene expression in mammalian cells was reported[5]. Recently, siRNA has gained widespread use as a tool to achieve sequence-specific inhibition of target gene expression *in vitro.*

However, recent data demonstrating that siRNAs can elicit off-target effects has raised a growing concern as to the specificity of these compounds[6]. These effects, which are unrelated to target gene inhibition, could lead to erroneous interpretation of RNAi experiments, or severely reduce efficacy of dsRNA therapeutic compounds. The use of short dsRNAs for *in vivo* applications is further limited due to lack of stability in serum. To address some of the limitations of traditional siRNAs, we have developed Stealth™ RNAi second-generation RNA chemistry for *in vitro* and *in vivo* applications. Stealth™ RNAi: (1) increases specificity by allowing only the antisense strand to efficiently enter the RNAi pathway (2) eliminates induction of interferon / PKR pathways (3) provides superior stability in serum.

Embryonic stem (ES) cells, by virtue of their ability to continually self-renew and to differentiate into any cell type, hold great promise for the field of regenerative medicine. These cells also provide a system for studying the underlying molecular mechanisms that control early development. A central challenge is to develop methods to direct differentiation of ES cells in a controlled manner to produce individual populations of specific cell types. Elucidation of molecular pathways that define ES cell pluripotency, self-renewal, and differentiation will be criticial to achieving this goal. We have developed molecular tools, along with transfection reagents and protocols to facilitate the regulation of gene expression in stem cell systems. Here we demonstrate the power of RNAi to silence genes responsible for key developmental pathways, to direct differentiation of stem cells. We have targeted OCT4, a known regulator of pluripotency. Levels of OCT4 expression govern embryonic stem cell fate. Upregulation is associated with commitment to extra-embryonic endoderm and mesoderm, while downregulation leads to formation of trophectoderm[7]. Here we show that Stealth™ RNAi can be efficiently delivered to mouse embryonic stem cells (mESC) to downregulate OCT4 expression in a specific manner, with subsequent induction of markers of trophectoderm differentiation. We also demonstrate the efficacy of Stealth™ RNAi in embryonal carcinoma (EC) cells, which provide a convenient system for validating molecular tools for application in ES cell models.

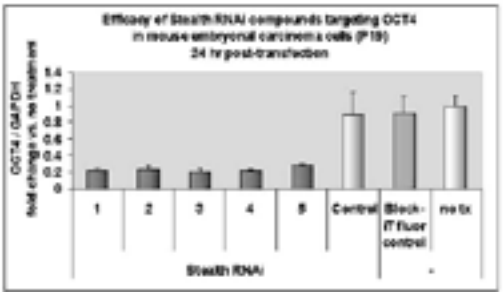

Figure 1. Inhibition of OCT4 expression by Stealth™ RNAi. P19 EC cells were transfected with 100 nM Stealth™ RNAi complexed with Lipofectamine™ 2000. A series of five Stealth duplexes were tested. After 24 hr, cells were then lysed and poly-A+ mRNA harvested. Equivalent amounts of mRNA were subjected to RT-qPCR analysis to quantify expression of the target and internal control (GAPDH) gene. arget gene expression is normalized to GAPDH and is presented as the fold change in expression relaive to no treatment.

Materials and Methods

Cell culture

P19 cells (ATCC CRL-1825) were maintained in Alpha Minimum Essential Medium (GIBCO 12571-048) supplemented with 7.5% bovine calf serum, 2.5% fetal bovine serum, 1.5 g / L sodium bicarbonate, penicillin / streptomycin. Mouse embryonic stem cells (mESC), D3 (ATCC CRL-1934) were maintained on mouse embryonic fibroblast (MEF) feeder layers treated previously with mitomycin C. mESC were cultured in 12-well plates in serum-free medium (KSR Complete Medium) consisting of KnockOut™ D-MEM (GIBCO) supplemented with L-glutamine, non-essential amino acids, β-mercaptoethanol, penicillin / streptomycin, leukemia inhibitory factor (LIF, ESGRO, Chemicon) and 15% KnockOut™ Serum Replacement (KSR, GIBCO). LIF was present in the media throughout cell culture and transfections.

Stealth RNAi

Five Stealth™ RNAi duplexes (Invitrogen) were designed to OCT4 (NM_013633) using the BLOCK-iT™ RNAi Designer at www.invitrogen.com/rnaidesigner. Gene silencing was quantified by RT-PCR following transfection in mESC and P19 cells.

Transfections

P19 cells were plated in 48-well plates 24 hr prior to transfection with 100 nM Stealth™ RNAi or 100 nM BLOCK-iT™ Fluorescent Oligo complexed with 2 µg / ml Lipofectamine™ 2000 (Invitrogen). Transfections were performed in triplicate for each treatment. After 24 hr, transfection efficiency was assessed by visualizing uptake of BLOCK-iT™ Fluorescent Oligo using fluorescence microscopy. Poly-A+ mRNA was isolated from individual wells of cells using the mRNA Catcher™ (Invitrogen).

mESC were transfected with complexes consisting of 50 nM BLOCK-iT™ Fluorescent Oligo or 50 nM Stealth™ RNAi and 2 µg / ml Lipofectamine™ 2000. mESC maintained on feeder layers were trypsinized, pelleted by centrifugation, and resuspended by trituration into a single cell suspension. mESC were then combined with freshly prepared transfection complexes and plated down onto fresh monolayers of MEF. Twenty-four hours later, uptake of the BLOCK-iT™ Fluorescent Oligo was assessed by fluorescence microscopy. Total RNA from transfected and non-treated wells of cells was harvested using the Micro-to-Midi™ Total RNA Purification System (Invitrogen).

RT-PCR analysis

qRT-PCR analysis on equivalent amounts of mRNA or DNAse-treated total RNA was performed using the Invitrogen SuperScript™ III Platinum® Two-Step qRT-PCR Kit and LUX™ primers (Invitrogen). For each sample, expression of target and marker genes was standardized to expression of GAPDH. qRT-PCR assays were performed in duplicate for each sample.

Immunocytochemistry

P19 cells were grown on glass coverslips for 24 hr prior to transfection with Stealth™ RNAi. After 24 hr, transfection media was replaced with fresh growth media and incubation continued for another 24

hr. Cells were then washed, fixed in paraformaldehyde, permeabilized with Triton X-100, blocked, and incubated with primary anti-OCT4 polyclonal antibody (Santa Cruz sc-8628) followed by secondary antibody (Alexa-Fluor 488 labeled rabbit anti-goat IgG, Molecular Probes). Immunocytochemical localization of OCT4 protein was visualized by fluorescence microscopy.

Results and Discussion

Transfections. Transfection of P19 EC cells and mESC with Lipofectamine 2000 was first optimized using the BLOCK-iT™ Fluorescent Oligo. Strong intracellular fluorescent signal was observed in a large proportion of the P19 cells following transfection. Likewise, mESC were efficiently transfected at 24 hr following co-plating of mESC and transfection complexes onto feeder layers. A high percentage of mESC colonies were visibly transfected, with strong fluorescent signal localized within individual cells in each colony.

Establishing optimal transfection conditions is critical to achieving high levels of gene knockdown in RNAi experiments. BLOCK-iT™ Fluorescent Oligo provides a convenient means to visually monitor the efficiency of transfection of a broad range of cell types. Once optimal transfection conditions have been established for a given culture system, we recommend including the BLOCK-iT™ Fluorescent Oligo to verify transfection efficiency in every RNAi experiment.

Target gene knockdown

To provide proof-of-principle for effective gene knockdown in an ES cell system, we targeted OCT4 (encoded by Pou5f1), a known regulator of pluripotency that controls lineage commitment in ES cells[7]. Following optimization of transfection conditions, we first conducted screens in P19 cells to identify active Stealth™ RNAi targeting OCT4. P19 EC cells are multipotent and express stem-cell specific genes, such as OCT4. They are relatively easy to culture and transfect, and provide a convenient system for testing efficacy of RNAi compounds against stem cell targets.

Four of five Stealth™ RNAi duplexes targeting OCT4 inhibited mRNA expression by ≥75% relative to the control (Fig. 1). The two best duplexes (1 and 3) targeting non-overlapping sites, were carried forward in experiments to downregulate OCT4 expression in mESC.

To determine the effect of Stealth™ RNAi on expression of OCT4 protein, we performed immunocytochemical analysis on P19 cells transfected with Stealth™ RNAi. At 48 hours post transfection, OCT4 protein was largely undetectable in cells transfected with OCT4-targeted Stealth™ duplexes, but was visibly localized to nuclei in cells transfected with non-targeted Control Stealth™ duplexes or in untreated cells (data not shown).

Transfection of mESC with Stealth™ RNAi resulted in ~ 75% inhibition of OCT4 expression at 24 hours by each of two Stealth™ duplexes

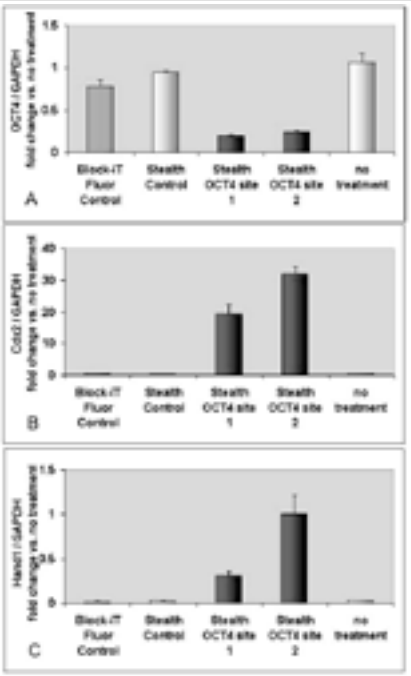

Figure 2. Induction of expression of trophectoderm makers Cdx2 and Hand1 following inhibition of OCT4 expression by Stealth™ RNAi. Individual wells of mESC were transfected with Stealth™ RNAi targeting two sites along the OCT4 mRNA. After 24 hr, cells were then lysed and total RNA harvested. Equivalent amounts of DNAse-treated total RNA were subjected to RT-qPCR analysis to quantify expression of target, marker, and internal control (GAPDH) genes. Target and marker gene expression is normalized to GAPDH and is presented as the fold change in expression relative to no treatment.

targeting non-overlapping sites (Fig. 2A). No appreciable change in OCT4 expression was detected in control samples relative to no treatment. MEF did not contribute to OCT4 expression, as expression was not detectable in these cells (data not shown). Induction of expression of two markers of trophectoderm differentiation (Cdx2 and Hand1) was detected only in those samples where OCT4 expression was inhibited (Fig. 2B & C). Expression of Cdx2, an early marker of trophectoderm differentiation[8], was induced ~20-30-fold at 24 hr. Induction of Hand1 expression, an intermediate marker[8, 9], was detectable at 24 hr. These results are consistent with previous reports demonstrating induction of trophoblast genes in response to down-regulation of OCT4 in ES cells[7, 10, 11]. Our results indicate that mES cells maintained in a serum-free co-culture system can be efficiently transfected with Stealth™ RNAi to achieve high levels of gene silencing, in order to dissect signaling pathways or induce differentiation in a specific manner.

Conclusions

Downregulation of OCT4 induces differentiation to trophectoderm. By using Stealth™ RNAi next generation RNA chemistry, we were able to effectively knock down OCT4 in mESC and detect expression of two trophectoderm markers, thus demonstrating the potential for RNAi as a tool for directed differentiation of stem cells. The ability to quickly and efficiently perform loss of function experiments using RNAi in EC and ES cell systems should facilitate progress in many areas of stem cell research.

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Advances in Stealth™ RNAi Design for RNA interference

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Abstract

RNA interference (RNAi) is an evolutionary conserved cellular process that has become a powerful new research tool to mediate sequence-specific gene silencing in a wide variety of mammalian cell types and whole organisms. Achieving the highest quality RNAi results requires the design of the most effective and specific RNAi molecules. Stealth™ RNAi, a chemically modified synthetic dsRNA, was developed to achieve high specificity and greater stability compared to unmodified siRNA molecules. To identify the optimal Stealth™ RNAi sequences for any target gene we developed the highly effective BLOCK-iT™ RNAi Designer. Through additional computation analysis of 1000 functionally validated Stealth™ RNAi sequences we were able to identify additional sequence specific characteristics unique to highly effective Stealth™ molecules. These features were subsequently integrated into the BLOCK-iT™ RNAi design algorithm and combined with an additional sequence specificity search to identify Stealth™ RNAi sequences most genes in the human genome (www.invitrogen.com/RNAiExpress). These newly designed Stealth™ Select molecules were highly effective in mediating gene silencing following transfection of A549 cells.

Introduction

RNA interference (RNAi) is valuable tool to identify genes and gene families involved in physiological and pathological processes [1]. One way that RNAi can be effectively achieved in mammalian cells is through the direct introduction of short interfering dsRNA (siRNA). Following transfection, one strand of the siRNA is incorporated into each RNA-Induced Silencing Complex (RISC) and guides the selection and site-specific cleavage of complementary target RNAs [2]. Each target RNA is rapidly degraded following RISC cleavage by intracellular exonucleases, effectively eliminating any functional activity.

Although RNAi can significantly reduce the expression of target RNAs, gene-silencing is often incomplete and can be associated with non-specific or off-target effects. These inherent features have led to the development of new chemical modifications and target selection strategies to maximize the RNAi response while reducing unwanted side effects. Stealth™ RNAi, for example, are 25nt, blunt-ended dsRNAs containing chemical modifications specifically designed to enhance serum stability, reduce sense strand mediated off-target effects, and prevent non-specific activation of stress response pathways. Collectively, these modification-mediated enhancements ensure more effective and clear RNAi results both *in vitro* and *in vivo*.

The efficiency and specificity of siRNA and Stealth™ RNAi mediated gene silencing is dependant on target site selection. Rigorous comparative sequence analysis of the target gene with other known transcripts is critical in maximizing RNAi design since non-targeted messages containing as few as 11 consecutive sequence matches with an siRNA can be effectively silenced [3]. Although RNAs

subsequently selected from the identified unique regions are expected to have a reduced chance of targeting another transcript, they often widely vary in effectiveness. Advances and improvements in the identification of highly effective RNAi sequences have recently emerged from bioinformatic sequence analysis of effective and ineffective siRNAs. Here we describe recent advances in improving Stealth™ RNAi design for increased efficacy and reduced unwanted off-target effects.

Materials and Methods

Stealth™ RNAi

Stealth™ RNAi were designed using the BLOCK-iT™ RNAi designer at http://rnaidesigner.invitrogen.com.

pSCREEN-iT™ Vector Preparation

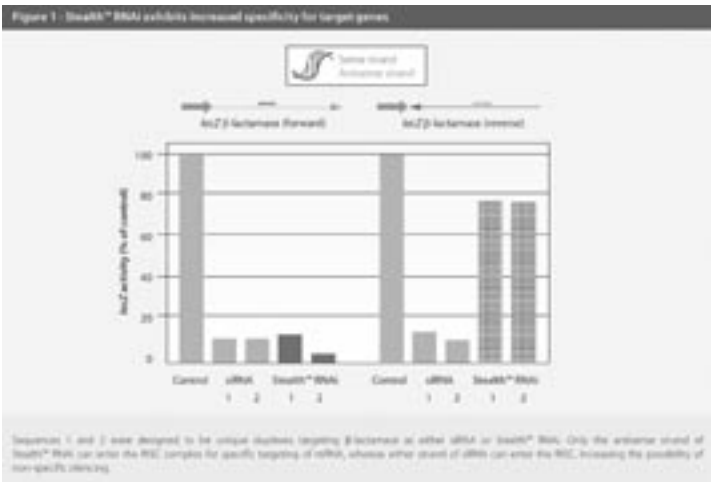
DNA from each Ultimate™ ORF entry clone was isolated using the PureLink™ 96 HQ Plasmid Miniprep Kit and quantified using Hoechst 33258 dye intercalation in 96 well plates. pScreen-iT™ expression vectors were subsequently generated in HTP using Gateway® recombination technology using pSCREEN-iT™/lacZ-GW/DEST DNA and each corresponding Ultimate™ ORF entry clone. Products of the LR reaction were transformed into Mach1™-T1 *E. coli* for amplification and plasmid DNA was subsequently isolated using the PureLink™ 96 HQ Plasmid Miniprep Kit and quantified using Hoechst 33258 dye intercalation. Sequence verification of all expression plasmids was performed using the following forward: (5′- ATTGGTGGCGACGACTCCTG –3′) and reverse (5′- ACCCGTGCGTTTTATTCTGTC -3′) sequencing primers.

pSCREEN-iT™ Target Screening Analysis

GripTite™ 293 MSR cells were cultured in DMEM containing 10% FBS, 0.1 mM NEAA, and 600ug/ml Geneticin®. Stealth™ RNAi were screened in Grip Tite™ 293 MSR cells in 96 well plates following transfection with 20ng pSCREEN-iT™ expression vector, 80ng pEF1a-Emerald vector, 0.2 pmol Stealth™ RNAi , and 0.5 ul Lipofectamine™ 2000 in a final volume of 100ul. Medium was changed for each after 3 to 5 hr of incubation at 37°C. Approximately 24 hours post-transfection the media was removed and replaced with 100ul of HBSS containing Ca^{2+} and Mg^{2+}. The expression of Emerald protein was determined as described below, and the HBSS was replaced with 100ul of cell lysis buffer. The cells were subsequently frozen at -70°C for at least 30min.

Reporter Detection

Emerald L64F GFP fluorescence was detected in cell monolayers plated in black side wall, clear flat bottomed 96 well plates. Briefly, the media was replaced with HBSS containing Ca^{2+} and Mg^{2} prior to detection to remove fluorescent molecules present in the growth media. Cellular fluorescence was detected with a bottom well detection at 509nm using an excitation at 487nm and a 495nm filter.



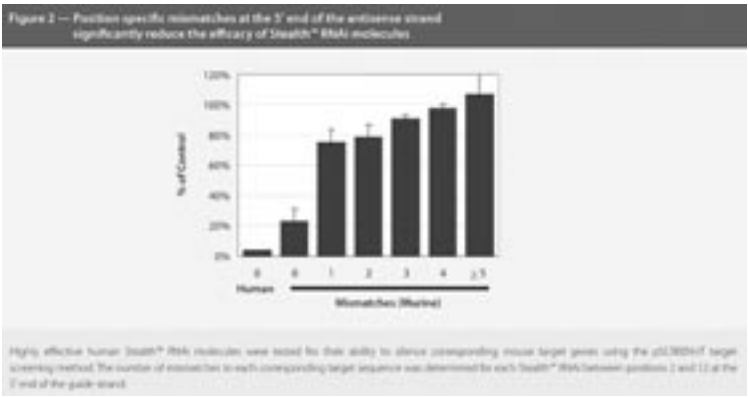
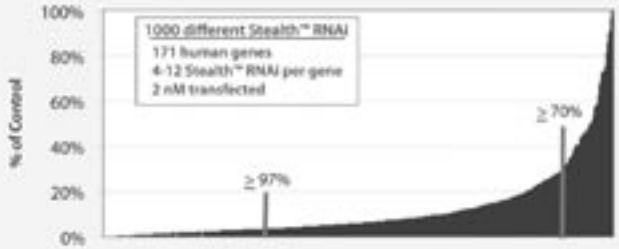


Figure 2 — Position specific mismatches at the 5' end of the antisense strand significantly reduce the efficacy of Stealth™ RNAi molecules

Highly effective human Stealth™ RNAi molecules were tested for their ability to silence corresponding mouse target genes using the pSCREEN-iT target screening method. The number of mismatches in each corresponding target sequence was determined for each Stealth™ RNAi between positions 2 and 12 at the 3' end of the guide strand.

Figure 3 – Highly effective Stealth™ RNAi design with the BLOCK-iT™ RNAi Designer



1000 Stealth™ RNAi duplexes were designed to 171 human genes. The ability of these sequences to knockdown a specific gene was measured using the BLOCK-iT™ RNAi Target Screening System in GripTite™ 293 MSR cells. Stealth™ RNAi were transfected at a concentration of 2 nM using Lipofectamine™ 2000. Over 90% of the sequences resulted in at least 70% knockdown, and 30% of these sequences showed greater than 97% knockdown.

Fluoresence measurements were measured using a SpectraMaX Gemini EM plate reader (Molecular Devices).

All frozen cell samples were thawed on a rotator at room temperature for at least 30min, and aliquots were assayed directly for β-galactosidase activity using the lacZ/Galactosidase Quantitation Kit (Molecular Probes, F-2905) according to the manufacturer's guidelines. Reaction fluorescence was detected with a bottom well detection at 448nm using an excitation at 390nm and a 435nm filter.

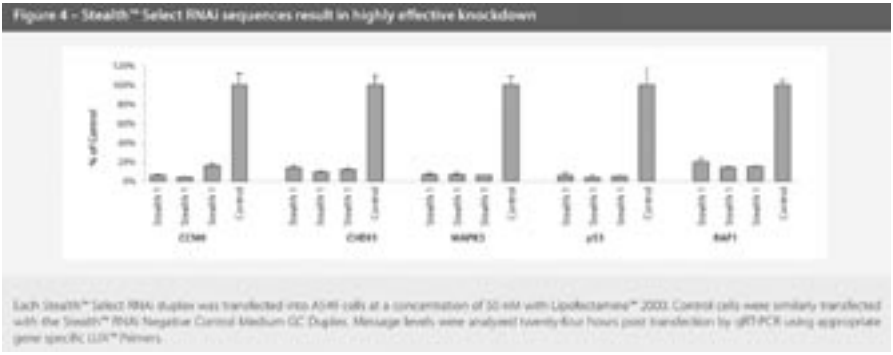
A549 Transfection and qPCR Analysis

A459 cells were transfected with 50nM of each respective Stealth™ RNAi using Lipofectamine™ 2000. Twenty-four hours following transfection, the cells were lysed and mRNA was isolated. qRT-PCR analysis was performed using target gene specific LUX™ primers with similar amounts of cDNA. Results for each sample were normalized to GAPDH.

Results and Discussion

Optimal RNAi design is achieved when the efficacy and specificity of gene silencing are both maximized. Stealth™ RNAi is uniquely chemically modified to enhance specificity by blocking the silencing activity of the sense strand (Figure 1). In contrast, both strands of an unmodified siRNA duplex can enter the RISC, allowing the sense strand to participate in unwanted off-target effects.

To further minimize potential off-target effects mediated by the guide strand, we sought to understand the individual contribution of position specific mismatches between the antisense strand and the target RNA. We selected 34 highly effective human Stealth™ RNAi sequences and tested their ability to reduce the expression of corresponding murine target genes using the pSCREEN-iT™ target screening system. Of this set, 3 sequences displayed 100% sequence identity to the mouse targets and demonstrated high activity. The remaining Stealth™ RNAi sequences contained one or more mis-



matches to the corresponding murine target gene. The position of these mismatches were compared with the level of gene silencing that was observed. Mismatches corresponding to positions 2-12 from the 5′ end of the antisense strand were found to markedly reduced the knockdown efficiency, consistent with previous observations using siRNAs [4] (Figure 2). Using the information identified in the cross-species analysis of Stealth™ RNAi, we developed an enhanced specificity search that including a Smith-Waterman sequence alignment to identify the most selective Stealth™ RNAi molecules.

The efficacy of 1000 Stealth™ RNAs designed using the BLOCK-iT™ RNAi Designer to 171 genes was determined by functionally testing using the pSCREEN-iT™ target screening system. The results of this study clearly demonstrates that the BLOCK-It™ RNAi Designer is very effective in selecting potent Stealth™ RNAi sequences with over 90% of the designed Stealth™ mediating 70% knockdown (Figure 3). Stealth™ RNAi sequences present in the most highly effective and least effective functional classes were further analyzed to identify specific design features uniquely present in highly effective Stealth™ RNAi. These features were subsequently used to further refine the Stealth™ RNAi design algorithm to improve design efficacy.

By combining the advanced algorithm and specificity search we designed 3 non-overlapping Stealth™ RNAi molecules to most genes in the human genome. Designed Stealth™ Select RNAs were subsequently tested for efficacy in mediating gene silencing. Stealth™ RNA were transfected into A549 cells at 50nM using Lipofectamine™ 2000. Target gene expression was analyzed twenty-four hours later using qRT-PCR. Each of the Stealth™ Select sequences tested was highly effective in mediating gene silencing often achieving 80 to 90% knockdown (Figure 4).

Conclusions

Stealth™ RNAi is a chemically modified dsRNA with enhanced specificity, stability, and reduced non-specific effects. Using the highly effective BLOCK-iT™ RNAi designer, thousands of Stealth™ RNAi molecules have been designed and functionally validated. Unique sequence features present in the most active and least active Stealth™ RNAi sequences were identified using computational analysis and integrated into the design algorithm. Using this algorithm and an advanced specificity search algorithm we have identified 3 non-overlapping Stealth™ Select RNAi molecules for most genes in the human genome. For more information on Stealth™ Select visit www.invitrogen.com/RNAiExpress.

www.invitrogen.com/rnai

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A 19th century monk fiddles with pea-pods in a monastery garden. One hundred and fifty years later the entire human genome is decoded.

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