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Defining and Assaying RNAi in Mammalian Cells

Perspective

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The investigation of protein function through the inhibition of activity has been critical to our understanding of many normal and abnormal biological processes. Until recently, functional inhibition in biological systems has been induced using a variety of approaches including small molecule antagonists, antibodies, aptamers, ribozymes, antisense oligonucleotides or transcripts, morpholinos, dominant-negative mutants, and knockout transgenic animals. Although all of these approaches have made substantial advances in our understanding of the function of many proteins, a lack of specificity or restricted applicability has limited their utility. Recently, exploitation of the naturally occurring posttranscriptional gene silencing mechanism triggered by double-stranded RNA (dsRNA), termed RNA interference (RNAi), has gained much favor as an alternative means for analyzing gene function. Aspects of the basic biology of RNAi, its application as a functional genomics tool, and its potential as a therapeutic approach have been extensively reviewed (Hannon and Rossi, 2004; Meister and Tuschl, 2004); however, there has been only limited discussion as to how to design and validate an individual RNAi effector molecule and how to interpret RNAi data overall, particularly with reference to experimentation in mammalian cells. This perspective will aim to consider some of the issues encountered when conducting and interpreting RNAi experiments in mammalian cells.

RNA Interference

In mammalian cells, RNAi can be triggered by a variety of dsRNA or dsRNA domain-containing molecules that are processed by the endoribonucleases Drosha and Dicer. Drosha is responsible for the processing of endogenously expressed RNAs with dsRNA domains, principally micro RNA (miRNA) precursors, in the nucleus (Bartel, 2004). Dicer further processes these precursors in the cytoplasm to yield miRNAs. Dicer is also responsible for the processing of other dsRNA molecules from exogenous sources into small duplex RNAs, with characteristic 2–3 nucleotide (nt) 3′ overhangs termed small interfering RNAs (siRNAs) (Bernstein et al., 2001; Elbashir et al., 2001b; Zhang et al., 2004). Dicer-processed RNAs, possibly still associated with Dicer, are unwound, and one strand of the duplex is incorporated into a ribonucleoprotein complex (RNP) containing members of the Argonaute (Ago) family of proteins (Hammond et al., 2000, 2001; Nykanen et al., 2001; Pham et al., 2004). In the case of siRNAs, this RNP complex has been termed the RNA-induced silencing complex (RISC) (Hammond et al., 2000; Hammond et al., 2001).

To date, at least two distinct, though not mutually exclusive, mechanisms have been described by which mi-RNP (miRNA-associated RISC) (Hutvagner and Zamore, 2002; Mourelatos et al., 2002) and siRNA-containing RISCs can mediate RNA silencing. Where there is complete or near-complete sequence complementarity between the small RNA and the target, the Argonaute 2 component of RISC mediates the cleavage of the target transcript at a site corresponding to 10 nts from the 5′ end of the single-stranded siRNA (Elbashir et al., 2001b; Liu et al., 2004; Meister et al., 2004). In contrast, miRNPs predominately appear to repress translation, with a key feature of the interaction being sequence mismatches between the miRNA and the target transcript (Kim et al., 2004; Olsen and Ambros, 1999; Seggerson et al., 2002).

Additionally, RNAi-associated processes have also been linked with inhibition of gene expression as a result of transcriptional gene silencing. Pioneering studies in fission yeast have shown that endogenously expressed repeat-associated siRNAs (rasiRNAs) incorporated into a Argonaute containing ribonucleoprotein complex, the RNA-induced initiation of transcriptional gene silencing complex (RITS), mediate chromatin modification through triggering DNA methylation (Verdel et al., 2004). Studies have also shown that siRNAs can induce DNA methylation in mammalian cells through the targeting of promoter regions (Kawasaki and Taira, 2004; Morris et al., 2004). At this early stage in our understanding of RNAi and its use to generate phenotypes for determining protein function, it is important not to rule out the possibility that RNAi mediated through protein translational repression or genomic modification may also be playing a role in mediating gene-specific silencing and any derived RNAi phenotype. Currently, though, most experiments using RNAi to examine protein function in mammalian cells have aimed to utilize exogenous RNAi effectors that are assumed to induce sequence-specific transcript cleavage.

Two broad categories of RNAi effector molecules have been developed to utilize the RNAi pathway so as to avoid triggering mammalian-specific dsRNA-associated proteins that mediate nonspecific effects in gene expression. Short hairpin RNAs (shRNAs) are single-stranded RNA molecules that possess intramolecular dsRNA domains. Synthetic siRNAs are RNA duplexes formed from two complementary but independent strands. Ideally the choice of RNAi effector molecule (e.g., whether to use a synthetic siRNA or shRNA) should be governed purely on the basis of the biological question under analysis. However, inevitably other practical factors (i.e., cost) have to be considered as well in designing RNAi experiments. Nevertheless, regardless of the choice of RNAi effector, it is critical that the molecule

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be designed to give the highest likelihood of generating an effective and specific RISC and that appropriate assays are conducted to confirm that a sequence-specific RNAi is responsible for the observed phenotype.

The Generation of Effective and Specific RISC

Asymmetric Loading of the Antisense siRNA Strand

There are a number of steps in the generation of a siRNA-associated RISC that could contribute to the overall efficiency with which a given complex can mediate sequence-specific cleavage of the target transcript. Biochemical and functional studies have identified some sequence-dependent features of siRNAs that appear to be critical to the overall efficiency of RNAi (Khvorova et al., 2003; Schwarz et al., 2003). Although these studies focused on synthetic siRNAs, these features should equally apply to siRNAs derived from shRNAs as well. Analysis of both miRNAs and effective synthetic siRNAs have shown that whichever strand is most easily unwound in a 5′–3′ direction will be preferentially assembled within RISC. There are also reports of increased probabilities of certain nucleotide combinations, including a relatively high stability for nts 5–10 (5′-3′ antisense single-stranded siRNA or guide strand) and particular nucleotides at specific positions. However, the mechanistic significance of these observations has not been established (Khvorova et al., 2003; Reynolds et al., 2004).

In Silico Identification of siRNA Sequences

A number of academic and commercially affiliated (freely accessible and proprietary) algorithms have been developed to assist researchers in the identification of efficient and utilizable siRNA sequences. In choosing a computational analysis tool, careful evaluation should be made as to what criteria are used, including when the tool was most recently updated and to what extent the tool has been experimentally validated. Nevertheless, any sequence(s) obtained from computer analysis should be judged as only having an increased probability of acting as a more effective siRNA when compared to other sequences that fulfill less of the specified criteria. Similar to the experience with oligonucleotide primers in PCR and sequencing technology, only experimental evaluation of multiple sequences will correctly establish which sequences are the most effective against a particular target transcript.

Target Transcript Variation

Genomic sequence diversity is a key feature of biological systems, and this variation must be considered at a transcriptional level. Single nucleotide polymorphisms (SNPs) can be found on average once every 300–500 bases in the human genome (Cargill et al., 1999; Nelson et al., 2004), and thus genetic polymorphism may affect RNAi effector sequence designs. The annotated RNA sequence for a particular gene may be a good starting point for the choice of a particular RNAi effector sequence, but it may also be important to consider the frequency and position of SNPs. Even different generations of the same cell line or strains (substrains) of inbred mice will differ in sequence either as a result of germline or somatic sequence changes. A potential consequence of studying transcripts with relatively high rates of polymorphisms within mammalian populations is that a particular RNAi effector of a given sequence that works in one biological context, e.g., a RNAi effector that works in one cell line, may not work as effectively in a different cell line. Care should also be made as to take note of the presence of alternative splicing, tissue- or tumor-specific transcripts.

The Potential for Off-Target RISC-Transcript Interactions

The efficiency with which a mismatched RISC can mediate transcript cleavage (or potentially a miRNP-like interaction resulting in translational repression) is probably significantly reduced as compared to when there is total complementarity (Haley and Zamore, 2004). Yet, microarray analysis has revealed that the expression of a nontargeted transcript with as few as 11 consecutive nucleotide matches with a siRNA sequence can be downregulated (Jackson et al., 2003). These observations have led to concerns that anything less than optimal RISC-transcript interactions could permit RNAi against an unintended target or an "off-target" interaction, which may limit our ability to interpret a functional effect seen after RNAi. Based on the limited data available, bioinformatic analysis should aim to eliminate siRNAs that have consecutive matches of ~17 or 18 nts between the antisense siRNA sequence and an mRNA sequence. Wherever possible, consecutive matches of less than this between the 5′ end of the siRNA antisense strand (in particular nts 2–12) and an mRNA sequence should also be avoided (Haley and Zamore, 2004). Designs that favor the generation of RISCs containing the antisense strand of the siRNA should help to reduce the possibility of sense strand/nontarget transcript interactions.

Currently it is unclear to what extent off-target miRNP-like translational repression interactions occur as microarray analysis can only detect downstream changes in RNA levels after repression of protein translation. Nucleotide positions 2–8 of miRNAs have been shown to be critical for miRNA-mRNA target recognition (Doench et al., 2003; Doench and Sharp, 2004). However, for fully effective repression, multiple mRNP-transcript interactions may be required, which could reduce the possibility of this type of interaction being a significant source of off-target effects (Doench and Sharp, 2004), although single miRNA/target interactions have been described in plants (Chen, 2004; Poy et al., 2004). Nevertheless, studies of RNAi transgenic animals (Carmell et al., 2003; Kunath et al., 2003; Rubinson et al., 2003; Ventura et al., 2004) suggest that off-target effects may be minimal on a whole-organism basis and that in cell culture, minimizing the concentration of a particular RNAi effector may reduce the likelihood of an off-target effect.

Nonspecific dsRNA Responses

The use of a minimal amount of an RNAi effector may also reduce the possibility of inducing the nonspecific dsRNA responses that most mammalian cells exhibit. Mammalian cells have a number of nonsequence-specific responses triggered by dsRNAs including some involved in viral host defense. Key effector proteins of these responses are the protein kinase-dsRNA dependent (PKR), and 2′–5′ oligoadenylate synthetase. On the basis of the few studies available, the degree to which these molecules are fully activated by dsRNAs is at least in part influenced by the size and concentration of the dsRNA (Manche et al., 1992; Nanduri et al., 1998). Data

addressing the degree to which siRNAs and shRNAs can interact with and activate non-RNAi-associated dsRNA binding proteins is still limited. However, what studies have been performed suggest that careful attention needs to be paid to the transcription and intracellular processing of shRNAs so that the siRNA generated does not trigger nonspecific responses and that directly administered siRNAs should consist of a high-quality, size-homogenous population (Bridge et al., 2003; Pebernard and Iggo, 2004; Persengiev et al., 2004; Sledz et al., 2003). Although all the factors that can potentially influence the degree of RNAi seen against a specific transcript need to be considered, only experimentation will determine the relative efficacy and/or specificity of a given RNAi effector molecule.

RNAi Effector Molecules: Singles, Doubles, and Pools

Short Hairpin RNAs

In addition to the complementary siRNA sequences, shRNAs consist of a linker sequence (which must be removed by intracellular processing) and appropriate promoter and termination sequences (which may need to be partially represented within the internal siRNA sequence). Initially, shRNA expression vectors utilized RNA polymerase III promoter and termination sequences to express the hairpin transcript. More recently, with an improved understanding of miRNA expression and processing, it has been possible to modify the flanking sequences to enable RNA polymerase II expression and importantly to utilize sequences that enhance Drosha/Dicer processing (http://www.openbiosystems.com/expression_arrest_shrna_introduction_psm2.php). A key advantage of shRNAs resides in the ability to express these single transcripts from plasmid or viral-based expression vectors. An almost bewildering number of shRNA expression systems have been described, utilizing different promoter-termination combinations, linker sequences, and other regulatory elements, including those that can be used to induce spatial or temporal specific expression. In addition, a number of add-on features, such as mammalian selectable markers for long-term expression, the coexpression of marker transgenes from the same plasmid to ease identification of cells expressing the shRNA or the inclusion of "barcode DNA sequences" (Berns et al., 2004; Paddison et al., 2004) have been described, and nearly all the different viral vector systems available have been adapted to express shRNAs.

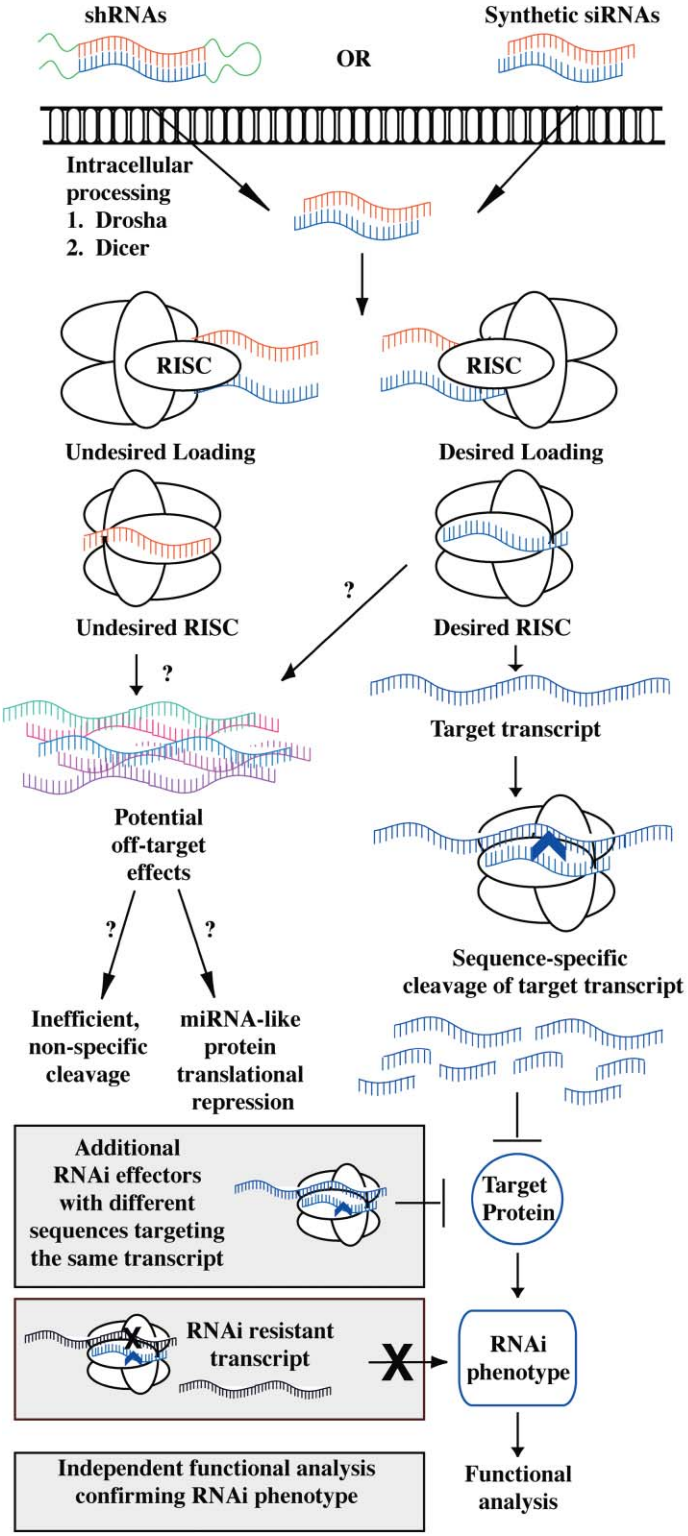
The choice of a particular shRNA expression system ultimately will depend on a number of factors, including the type of cells in which RNAi will be performed and the length of time for which RNAi expression is required. Practically, the fidelity of the shRNA sequence should be checked as oligonucleotide synthesis, cloning, and bacterial amplification and/or virus preparation all have the potential to induce sequence changes, which may influence both the efficacy and the specificity of gene silencing. Further, an appropriate level of expression and processing will be required that ensures that sufficient shRNA is expressed to induce RNAi, but not so much as to overwhelm the RNAi machinery. In this regard minimizing the copy number of integrated stably expressed shRNAs may be critical as each integration event could increase the potential for insertion within an active transcription unit, which in turn may influence the interpretation of any derived phenotype, though this may be a more significant issue for vectors based on murine leukemia virus than for lentiviral-based vectors (De Palma et al., 2004; Kustikova et al., 2003). The proper initiation and termination of transcription of the shRNA is also critical if correct processing is to occur and to minimize the generation of nonspecific dsRNA responses related to other mammalian dsRNA responses (Pebernard and Iggo, 2004).

Duplex RNAs

The first studies of RNAi in mammalian cells used chemically synthesized RNA oligonucleotides to mimic naturally occurring siRNAs (Caplen et al., 2001; Elbashir et al., 2001a). Synthetic siRNAs are a more defined reagent that can be subjected to standardized quality control procedures. Synthetic siRNAs are usually delivered using standard physicochemical transfection methods. The efficiency of transfection of synthetic siRNAs is dependent upon the method used and transfectability of a particular cell line. Initially, generic methods for delivery of larger nucleic acids were used for the siRNA, but more recent second generation protocols and reagents have been developed to improve the efficiency of siRNA introduction into the cell. The primary advantage in the use of synthetic siRNAs, assuming well-optimized transfection conditions, is the ability to control the amount of siRNA that is accessible for incorporation into RISC, which may minimize the possibility of off-target and/or nonspecific effects. Optimization of transfection conditions is best achieved using RNAi as an outcome measure. The use of surrogates such as fluorescently labeled synthetic siRNAs or other nucleic acids is likely to be only a broad indicator of the efficiency with which a particular set of conditions will work. The key experimental disadvantage of siRNAs is the transient nature of the silencing effect, which can impact studies when trying to inhibit expression of proteins with a long half-life or where the derived RNAi phenotype requires an extended period of inhibition of gene expression. Expressed siRNAs from complementary independent, tandem, or convergent expression cassettes have also been described but have not been as extensively utilized as shRNA-based systems.

Pools of RNAi Effector Molecules

In invertebrate species, pools of siRNAs targeting different regions of target RNA transcripts are generated intracellularly through Dicer-mediated processing of larger dsRNAs. Pools may be advantageous, as there is an increased probability of at least one highly effective siRNA being present within the population. Additionally, the decreased concentration of any one siRNA may reduce the potential for sequence-specific off-target effects. Endonuclease-generated siRNAs have been used to generate pools of siRNAs against mammalian genes as these have relatively low initial production costs (Kawasaki et al., 2003; Myers et al., 2003), but ultimately, individual siRNAs will still need to be identified and generated to fully validate a particular RNAi phenotype. The assessment of synthetic siRNAs and shRNAs in pools (corresponding to the same and/or different genes) has also been used as part of high-throughput screening efforts and as an approach to reduce potential "off-



Select the most appropriate RNAi effector molecule for the biological question under study

Minimize amount of RNAi effector used

Employ appropriate negative control(s) to assess for the effect of both the delivery system and the nucleic acid used, including intracellular proccessing steps

Use *in silico* analysis to increase the probability of the formation of an effective and specific RISC

Awareness of target transcript polymorphisms, and splice variants

Avoid extensive sequence homology between RNAi effector sequences and non-targeted transcripts, particularly at the 5' end of the RNAi effector sequence.

Generation of a sequence-specific, efficient cleavage competent complex

Measure changes in target mRNA levels as compared to an endogenously expressed control transcript.

Measure changes in target protein levels as compared to an endogenously expressed control protein. May require assessment at multiple time points

Replicate RNAi phenotypes using RNAi effectors of different sequence targeting the same transcript.

Rescue of RNAi phenotype using expressed version of target transcript resistant to RNAi and/or verification using independent methodology

Figure 1. Critical Steps in the RNAi Mechanism and Some Experimental Parameters Related to Them

target" effects (Hsieh et al., 2004). Realistically, detailed analysis of an RNAi phenotype in mammalian cells will ultimately, in most cases, require separate experiments using RNAi effectors targeting different sequences within the same transcript.

Is This RNAi? Molecular Assays of RNAi

The goal of most investigators utilizing RNAi is to study protein function through inhibition of gene expression. By generating a RISC against a particular RNA target, a decline in protein synthesis should occur after RNA

cleavage and degradation (Figure 1). A key question for investigators is what is required to enable confident interpretation that an observed phenotype is a direct consequence of sequence-specific RNAi against the target gene of interest. In the case of targeting noncoding transcripts (including in some viral RNA transcripts), RNA analysis alone is obviously appropriate. In the case of protein-encoding transcripts, however, a decrease in the level of both RNA and protein (normalized against appropriate controls) will be the clearest indication of effective RNAi through RISC-mediated cleavage of the targeted RNA transcript. It should be anticipated that there should be some, but not necessarily absolute, correlation between RNA and protein levels after RNAi. For example, a decrease in RNA levels not seen at a protein level at a particular time point could reflect protein stability. In such cases, investigators should obviously consider examination of additional time points. Furthermore, a change in protein levels that is not accompanied by a change in RNA levels may reflect RNA silencing through a protein translational blockade rather than through RNA cleavage and degradation. As we still understand little about the mechanistic differences between RISC cleavage and protein translational blockade, it would be highly advantageous to acquire information as to when RNAi phenotypes are a consequence of this type of interaction rather than RISC-associated cleavage. This will only be possible if both RNA and protein results are reported.

RNA Assays

Many different assays of intracellular RNA levels, including Northern blot, reverse transcriptase-PCR (RT-PCR)-based methods, branched-DNA assays, and microarray analysis can be utilized to examine the effects of RNAi on the transcript levels. To date and where presented, the analysis of RNAi at a RNA level is usually based on quantification of reverse-transcribed and PCR-amplified material. Ideally, any PCR-based assay should be designed to minimize the theoretical possibility of amplifying cleaved target RNA, through the use of PCR primers that flank the putative siRNA cleavage site. Any quantitative PCR procedure should be carefully optimized particularly for low abundance transcripts where a significant level of silencing may represent only a small change in the cycle threshold. Unlike protein analysis, the time point(s) at which RNA analysis is conducted is probably less critical. In most cases, assays performed between 36 and 72 hr after initiation of RNAi should give appropriate results irrespective of whether siRNAs or shRNAs are used. These types of assays assume the knockdown of the target gene is compatible with cell survival. Quantifying RNA or protein levels from actively dying cells may be difficult and thus careful monitoring of the expression of control genes may be required not only to normalize the assay but also to detect any gross changes in cellular state. An additional issue that needs to be considered is the steady-state levels of target RNA expression to ensure that there is a sufficient level of gene expression to confidently observe a downregulation.

Protein Assays

The vast majority of RNAi studies of protein-encoding transcripts employ standard antibody-based assays (principally nonquantitative Western Blot analysis) to analyze changes in the levels of proteins corresponding to RNAi-targeted transcripts. Intuitively, the half-life of a protein targeted through RNAi is critical for determining when to assay protein levels, as a reduction will only be observed following protein turnover in the absence of new protein synthesis. Furthermore, in order to observe a decrease in the level of a long-lived protein, stable silencing through the use of a shRNA may be more desirable than employing a short-lived synthetic siRNA. As cell cultures are typically asynchronous, an additional source of variation in protein expression may arise from cell cycle-associated differences. Perhaps the greatest challenge to analyzing RNAi at a protein level is the lack of well-characterized antibodies, and this may become an increasingly significant issue as RNAi is used more extensively to study genes for which there is no or only minimal experimental data.

Surrogate Assays

A number of surrogate assays for RNAi against endogenous genes have been developed. For example, the cotransfection of plasmids expressing the target transcript of interest with or without fusion to a marker gene and an RNAi effector may be a useful screening tool, particularly as it allows analysis of genes that otherwise might be difficult to study, for example, genes with low or restricted expression patterns (Kumar et al., 2003). However, follow-up studies examining the inhibition of the endogenous transcript should also be attempted.

Phenotypic Assays and RNAi Experimentation

The Use of Multiple RNAi Effector Sequences per Target

In 1994, Stein and Kreig highlighted the issue of interpreting data derived from the use of antisense oligonucleotides and stressed the need to examine multiple sequences corresponding to different regions of the same transcript (Stein and Krieg, 1994). Similarly, it has been recommended that RNAi phenotypic changes, wherever possible, be confirmed using multiple RNAi effectors against the same target but with different sequences (Nat. Cell Biol., 2003, Vol. 5, 489–490). RNAi effectors (siRNA or shRNA) that induce a specific downregulation of a particular gene to a similar degree should broadly induce the same phenotype. However, it should be kept in mind that the activity of some proteins may be concentration dependent. Thus, small differences in the degree of downregulation mediated by different RNAi effectors against the same target may cause some subtle differences in the observed phenotype. In these circumstances several additional RNAi effector molecules should be studied. The true specificity of RNAi will only indisputably be established by using large-scale analysis, such as microarray and proteomic screens, where multiple gene and protein expression profiles can be analyzed. It is recognized, however, that such assays will only be practically possible in a limited number of studies. Reporting specificity for a particular RNAi effector when the expression of only one or two additional genes has been examined is probably not sufficient as a means for extrapolating specificity against all transcripts.

Experimental Controls

All methods that mediate the delivery of nucleic acids to mammalian cells can produce a negative, e.g., cytotoxic

Table 1. RNAi Terminology

RNA silencing	A general, species-independent term for the regulation of gene expression through the activity of a RNA intermediate.
RNA interference (RNAi)	Inhibition of gene expression requiring a dsRNA or dsRNA domain-containing molecule processed by a RNase III-like endonucleases and/or the generation of a ribonucleoprotein (RNP) complex containing a small RNA molecule and member(s) of the Argonaute (Ago) family of proteins.
RNAi phenotype	A phenotype generated as a result of the induction of RNAi.
RNAi effector	Any RNA molecule that can mediate an inhibition in gene expression through RNAi.
Small interfering RNAs (siRNA)	Small duplex RNA molecules from or derived from exogenous nucleic acid sources.
Repeat associated siRNAs (rasiRNAs)	Small, endogenous RNAs encoded by repeat sequences.
Micro RNAs (miRNAs)	Small RNA molecules processed from endogenously expressed transcripts (primary and precursor miRNAs) that form a stem loop intermediate.
RNA-induced silencing complex (RISC)	An Ago-containing RNP complex incorporating a single-stranded siRNA derived from an exogenous RNA source.
Micro RNA-protein complex (miRNP)	An Ago-containing RNP complex incorporating an endogenously derived single-stranded miRNA.
RNA-induced initiation of transcriptional gene silencing (RITS)	An Ago-containing RNP complex incorporating repeat-associated siRNA.
Single-stranded antisense or guide strand	The strand of the siRNA complementary to the target transcript and the favored strand for loading into effective RISCs for sequence specific cleavage.
Functional analysis using RNAi	An assessment of protein function through the use of RNAi -based methods, resources, and technologies. The assumed aim is that silencing is mediated through transcript cleavage using most of the approaches developed to date and that downstream phenotypes are as consequence of a reduction in the level of the target protein.
RNAi-genomic modification (RNAi-gm)*	RNAi as a result of genomic DNA modification.
RNAi-cleavage (RNAi-c)*	RNAi as a result of transcript cleavage.
RNAi-repression (RNAi-r)*	RNAi as a result of repression of protein translation.
siRNAs for functional analysis	RNA duplexes generated to structurally resemble naturally occurring siRNAs. Can be produced by (1) chemical synthesis, (2) through the action of endonucleases on dsRNA (sometimes termed esiRNAs), or (3) coexpression of single stranded RNAs (from independent, tandem or convergent expression cassettes) to generate a siRNA. Care should be made to clearly define the method of production.
Short hairpin RNAs (shRNAs)	An expressed hairpin RNA intracellularly processed to generate a siRNA.

*These subdefinitions do not assume a particular type of RNA trigger or an exact definition of the interaction between the small RNA molecule and the target nucleic acid, (e.g., perfect or imperfect sequence alignment).

effect on cells that in most cases is usually dose dependent. Critical to determining the correct control to eliminate this variable is an appreciation that the delivery vehicle alone may have a very different cytotoxicity profile to that of the delivery vehicle plus nucleic acid. For example, when a cationic lipid is used for the delivery of synthetic siRNA, it is essential that the control used consist of a complex of exactly the same amount of lipid and a negative control synthetic siRNA. Equally important, electroporation should also be conducted with a negative control synthetic siRNA handled under identical conditions to the experimental nucleic acid. In addition, a plasmid or viral vector carrying no expressing shRNA may induce minimal effects in comparison to one that expresses a negative control shRNA. An expressed negative control shRNA that is processed intracellularly should be a good test for the effects of utilizing the cell's transcription and RNAi processing machinery. An additional reason for the use of a negative control RNAi effector molecule, siRNA or shRNA, is to take account of the possibility that the exogenous molecule is saturating the RNAi machinery such that the processing of endogenous RNAs required for normal gene expression is disrupted. A key issue, though, is the choice of a negative control sequence. It is likely that lack of sequence homology is more important than the exact sequence composition of a negative control, but an overall thermodynamic profile similar to an active siRNA is probably desirable. Sequences that target nonmammalian marker transgenes may be particularly useful as these can be used as both a negative and positive control. Scrambled versions of the siRNA sequence corresponding to the target are probably not appropriate. The development of a set of universally defined and validated control sequences for use in mammalian cells would be a critical resource that should be considered.

Reporting an RNAi Phenotype

The description of protein function as a consequence of RNAi analysis is rapidly becoming a key component of biological studies. However, inconsistencies in the description of the RNAi-based methodologies and resources used, lack of full descriptions of the steps used to validate the RNAi effector molecules, the use of inappropriate controls, and the absence of independent biological assessment of the observed phenotype often make it difficult to draw valid conclusions about a reported phenotype. Technologies based on RNAi have developed very quickly, which in turn has led to the development of numerous terms for RNAi-related resources and methodologies. As the exact process used to mediate RNAi may be critical to the interpretation of an RNAi phenotype, we would like to advocate that a

Table 2. Assaying an RNAi Phenotype in Mammalian Cells

(1) Applicable to All RNAi Studies
Clear use of terminology to define the type of RNAi effector used.
Optimized delivery to minimize the amount of an individual RNAi effector used.
Reporting of all RNAi effector sequences.
The use of negative controls consistent with the mode of delivery and the type of RNAi effector used.
Consideration and clear reporting of the time point(s) at which assays of RNAi effects are conducted.
To fully characterize whether or not a particular RNAi effector acts through transcript cleavage, both RNA and protein levels should be presented with normalization to appropriate controls.
(2) Large-Scale RNAi Screens
Multiple RNAi effectors per gene target should be used to reduce the number of false positives.
Inclusion of negative and positive control siRNAs appropriate for the nucleic acid, the delivery method, and the assay used.
If "pooled" RNAi effector approaches are used, then follow-up secondary screens using more restricted pools or individual RNAi effector molecules may be required.
Precharacterization of individual RNAi effectors in a large-screen is not appropriate, but detailed analysis of small numbers of RNAi phenotype "hits" should be used to validate a large-scale approach as a whole.
(3) RNAi Analysis against Single or Limited Numbers of Genes
Analysis of multiple RNAi effectors of different sequence corresponding to the same transcript to define function unless clear confirmation of function using an alternative methodology is presented.
Rescue of RNAi phenotype and/or verification of RNAi phenotype using alternative methodology.
Wherever possible, a quantification of the degree of RNAi and any phenotypic changes should be presented to assess the correlation between them.
(4) In Vivo and Ex Vivo RNAi Analysis
(a) Precharacterization of the RNAi effector and delivery system in cell culture.
(b) Somatic Cell-Based RNAi
Ex vivo RNAi analysis
Consideration of the effect of the timing from the initiation of RNAi *ex vivo* to introduction into animals.
Route of administration of ex vivo-treated cells will be critical to the RNAi phenotype achieved, it may be appropriate to assess more than one.
Comparison of RNAi phenotype data to suitable negative controls that are consistent with the mode of delivery, RNAi effector, and any additional ex vivo manipulations (e.g. drug selection in cell culture or in vivo).
In vivo RNAi analysis
Consideration of the route of administration and the likely tissue and/or cell-type to be targeted.
Assessment of the degree of RNAi in all appropriate tissues and/or cell-types, where possible RNA and protein levels should be analyzed, as well as any phenotypic change.
(c) Germ-line-based RNAi
Constitutive expression
Assessment, including copy number, of the integration of viral DNA.
Assessment of RNAi in all appropriate tissues and/or cell-types. Where possible RNA and protein levels should be analyzed, as well as any phenotypic change. Quantification to determine the level of variation in the degree of RNAi within a whole organism may be critical for interpretation of an RNAi phenotype.
Spatial or temporal expression
Assessment, including copy number, of the integration of viral DNA.
Assessment of RNAi in all appropriate tissues and/or cell-types, where possible RNA and protein levels should be analyzed, as well as any phenotypic change. Quantification of the degree of RNAi to confirm spatial specificity and/or the level of RNAi will be critical for interpretation of an RNAi phenotype.

more standardized use of terms be encouraged when RNAi experimentation is reported. Listed in Table 1 are some commonly used RNAi-related terminology and some new suggestions we would like to put forward that could be used to clarify some of the admixture of terms. In particular, we would like to encourage adoption of a nomenclature that enables differentiation of RNA-mediated inhibition of gene expression through alterations at a genomic level, through transcript cleavage, or through repression of protein-translation. It may also be helpful to encourage the incorporation of existing standardized gene nomenclature into the terminology for individual RNAi effectors. For example, synthetic siRNAs or shRNAs to a particular gene should reflect origin (human, mouse, etc.) as well as nucleotide location at least for well-annotated transcripts, which may be a way of distinguishing between sequences targeting the same gene. For example, a short hairpin RNAi effector targeting the coding region beginning at nucleotide 342 of human *HRAS1* could read sh_HRAS1_342; alternatively, RNAi effectors could refer to the accession number of the transcript sequence used for the design of the siRNA. We would also like to recommend the establishment of centralized public RNAi effector sequence databases that would enable investigators to rapidly access sequences against a given transcript (perhaps linked, where available, to validation and functional data) without the need to search multiple sources as is required now.

The type and number of assays that should be conducted to validate an individual RNAi effector and/or a RNA phenotype will be context-dependent and evidence will, in most cases, need to be additive. Table 2 summarizes many of the issues that should be consid-

ered in RNAi experiments conducted in mammalian cells. To fully assert that catalytic RISC-mediated RNAi against a particular protein-encoding transcript is occurring, we advocate assaying both RNA and protein levels. In addition, the use of appropriate negative controls and multiple RNAi effectors of different sequence corresponding to the same target is critical for validation and interpretation of an RNAi phenotype, particularly if RNAi is the sole methodology used to establish function. The minimum number of RNAi effectors with different sequences targeting the same transcript that will need to be experimentally tested will probably be dependent on the biological question under investigation and the additional functional data presented. However, when an RNAi phenotype is the only criterion for the functional characterization of a particular protein, at least two sequences mediating broadly similar levels of inhibition at a molecular level should be assessed. Phenotypic changes mediated by RNAi should be rescuable by expression of a transgene expressing a cDNA resistant to the sequence-specific RNAi against the target (Nat. Cell Biol., 2003, Vol. 5, 489–490). This can be achieved experimentally by using transgene expression of a cDNA corresponding to the target gene that is resistant to RISC cleavage through, for example, the introduction of a silent mutation in the target sequence (Lassus et al., 2002). The use of cDNA corresponding to the ortholog of the protein in question may be advantageous in this regard as this may differ at the nucleotide level such that it is unaffected by an RNAi effector targeting the original species gene. Alternatively if the siRNA targets a 5′ or 3′ untranslated region, a cDNA without these regions can be used. However, this may not always be possible. In such cases and where only one sequence-specific RNAi effector per target transcript has been used, detailed confirmation of the RNAi phenotype changes using independent methods such as small molecule inhibitors, antibodies, or other means will be absolutely essential.

RNAi analysis in vivo sets some additional challenges and so prior validation of the RNAi effector in a suitable cell-based system will be critical as the type of molecular and phenotypic analysis possible in animals is somewhat different. RNAi has been applied to cells in culture that are then assessed in vivo, by direct administration of RNAi effectors in vivo to induce effects in somatic tissues or by RNAi induction in embryonic cells for the generation of transgenic animals (Hemann et al., 2003; Soutschek et al., 2004; Ventura et al., 2004; Xia et al., 2004). In all cases any aspect of the RNAi phenotype should be carefully compared to that seen in control animals, which should take into account the RNAi effector including any modifications for in vivo application, the delivery system, the amount delivered, and the route of administration.

There are a number of factors that can significantly influence an RNAi phenotype, including when an assay is conducted relative to initiation of RNAi, the degree of RNAi achieved, the threshold at which the decrease in protein levels mediates a response, and the context in which an experiment is conducted, (e.g., the cell line used). Functional redundancy and the upregulation of compensatory proteins or pathways may also substantially influence the generation and interpretation of an RNAi phenotype, particularly where long-term, stable expression of an RNAi effector has been utilized. In addition, simple or even complex feedback mechanisms favoring maintenance of the steady-state transcription of a mRNA targeted by RNAi may, through RNA or protein regulatory effects, act at such a rate that the kinetics of the RNAi effect are less favored than those of the regulatory effect, resulting in the failure to generate a functional RNAi phenotype. Such cellular compensatory effects may be particularly seen when long-term (over ~3 weeks, four to eight passages) suppression of the expression of functioning protein is under analysis. It must be emphasized that in most cases a cell under stress will attempt to compensate for the loss of an important protein, especially over long periods of time, and if unable to return to a steady-state of protein concentration, may undergo apoptosis-mediated cell death. Alternatively, subpopulations of cells with a lower level of RNAi against the target transcript (either originally or through, e.g., promoter repression) may have a growth advantage over other cells in the population in which RNAi is more effective. This would lead to the generation of selected populations of cells where the RNAi phenotype may be significantly altered from that seen at earlier time points.

The analysis of cellular processes through application of RNAi-based technologies has, and will continue to have, an enormous impact on our understanding of biological processes. As is often the case though, the complexity of mammalian systems requires that care be taken to ensure that an RNAi phenotype reflects a real functional activity. As more validation and downstream functional data is generated for individual RNAi effector molecules, it should be possible to see transcript sequence information matched with well-defined siRNA sequences that have been shown to generate a specific degree of knockdown in a variety of biological settings with a significant level of reproducibility. In the meantime, however, clarity in the presentation of RNAi experimentation will be critical if we are to ensure that RNAi phenotypes in mammalian cells become a reproducible means of elucidating protein function.

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