REVIEW ARTICLE

Optimizing RNA interference for application in mammalian cells

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Over the last 2 years, the scientific community has rapidly embraced novel technologies that allow gene silencing in vertebrates. Ease of application, cost effectiveness and the possibilities for genome-wide reverse genetics have quickly turned this approach into a widely accepted, almost mandatory asset for a self-respecting laboratory in life sciences. This review discusses some of the recent technological developments that allow the application of RNAi (RNA interference) in mammalian cells. In addition, the advantages of applying RNAi to study cell cycle events and the emerging approaches to perform mutational analysis by complementation in mammalian cells are evaluated. In addition, common pitfalls and drawbacks of RNAi will be reviewed, as well as the possible ways to get around these shortcomings of gene silencing by small interfering RNA.

Key words: complementation analysis, mitosis, off target, secondary effect, small interfering RNA (siRNA), synchronization.

INTRODUCTION

Gene silencing by RNAi (RNA interference) was first applied in plants and *Caenorhabditis elegans* to reduce gene expression of selected genes through the introduction of long dsRNA (double-stranded RNA) molecules homologous with the gene of choice (for a review, see [1]). The power of this approach became evident when it was demonstrated that introduction of a few copies of dsRNA is sufficient to virtually completely abolish expression of the complementary endogenous gene [2]. Even though the phenomenon of RNAi was initially poorly understood, it quickly 'caught on' in *C. elegans* genetics as a convenient tool to study gene function. Many reports demonstrated that RNAi by means of introduction of dsRNA in *C. elegans* could result in a phenocopy of the nullizygous mutation of that given gene, or alternatively, produce an allelic series of mutant phenotypes. The ease of application and powerful selectivity of RNAi turned *C. elegans* into a favoured model organism for reverse genetic approaches.

Since then, it has been demonstrated that RNAi is mediated by siRNAs (small interfering RNAs) that are produced from dsRNA through a cell-autonomous mechanism (reviewed in [3]). This latter mechanism turns out to be conserved in higher organisms. However, in cells of vertebrate origin, introduction of long dsRNAs triggers activation of the PKR (RNA-dependent protein kinase) pathway, leading to a very efficient shutdown of cellular protein synthesis (for a review, see [4]). These non-specific effects caused by activation of the PKR pathway initially severely hampered possible applications of RNAi in vertebrate cells. This problem was resolved when it could be demonstrated that the direct introduction of siRNAs, rather than a long dsRNA, is an effective method to knock-down expression of a given gene, while this evades the activation of the PKR pathway [5,6]. Thus the capacity to undergo RNAi has clearly been retained in higher organisms, but why did this mechanism arise in Nature in the first place? Recent work has made it evident that many organisms can use dsRNA-induced gene silencing systemically during development, for cellular protection against viruses, to prevent transposon jumping, or for silencing of introduced transgenes (for reviews, see [3,7,8]).

Over the last couple of years, a large variety of techniques have emerged that have made dsRNA-induced silencing easily applicable to cells of higher organisms (for a review, see [9]). Thus the irreversible change that took place in worm and plant genetics is now taking place in mammalian systems. One can be certain that RNAi will revolutionize reverse genetics approaches in mammalian cells, and that this technology will allow a dramatically accelerated progress towards understanding of gene function. However, the lack of amplification of the RNAi response does limit the efficacy of RNAi in higher organisms, and as a consequence RNAi produces a hypomorphic mutant at best. No matter how much protein expression is reduced after targeting of the gene of choice, a low residual amount of protein function can never be excluded. Because of this, excluding a function for that particular gene in a certain pathway is never fully warranted. However, it is very tempting to compare the relative contribution of a gene to one pathway or another. As a consequence, negative results will be included in the description of the overall RNAi phenotype and (carefully) interpreted as evidence that this gene is not required for certain aspects of cellular behaviour. In addition, even though the time required between interference with gene function and analysis of cellular behaviour is faster with RNAi than with most other technologies, results can still be perturbed by secondary effects, adaptation of the cells under investigation or toxicity of the procedure. This review addresses these issues and discusses some of the possible solutions to get around such problems.

THE EVOLUTION OF RNAi

More than a decade ago, several groups reported that the introduction of a transgene in plants could lead to silencing of the homologous endogenous gene [10]. This phenomenon was termed 'co-suppression', as it was shown that both the transgene as

Abbreviations used: dsRNA, double-stranded RNA; miRNA, micro RNA; PKR, RNA-dependent protein kinase; PTGS, post-transcriptional gene silencing; RdRP, RNA-dependent RNA polymerase; RISC, RNA-induced silencing complex; RNAi, RNA interference; pol III, RNA polymerase III; shRNA, short hairpin RNA; SID1, systemic interference defective 1; siRNA, small interfering RNA; stRNA, small temporal RNA; UTR, untranslated region.

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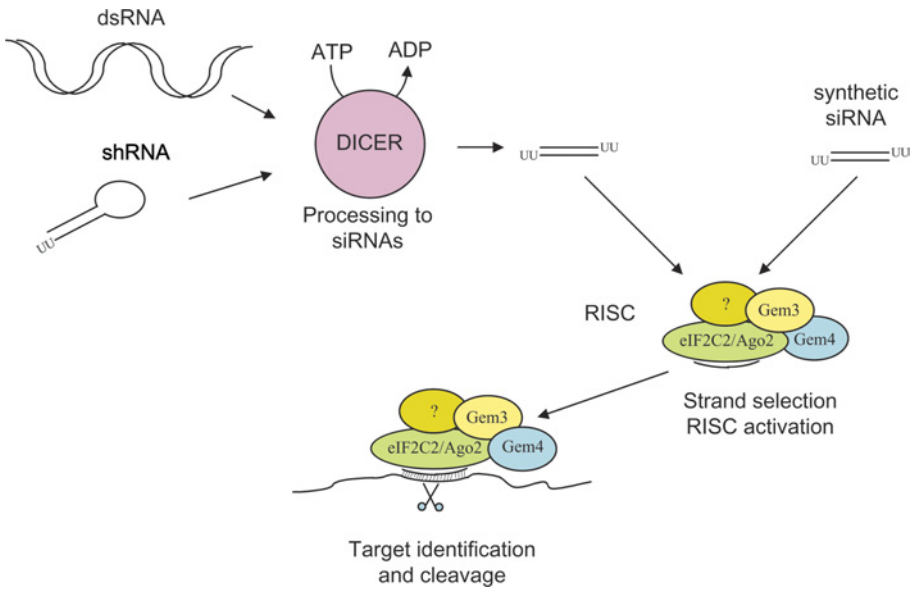

Figure 1 siRNA pathways

Long dsRNA or shRNAs are processed by Dicer, an enzyme containing ribonuclease III-like nuclease activity. This results in the generation of ≈22–25-nucleotides-long siRNAs, with two unpaired uridine nucleotides at the 3′ end of each strand. The two strands of the siRNA are unwound by the RISC, and one strand is selected to identify a fully complementary target mRNA. Strand selection is determined by the relative stability of base-pairing at the respective ends of the siRNA duplex (see text for details). RISC is a multi-subunit complex of which a number of components have been identified. In mammalian cells it contains a protein of the Argonaute family (eIF2C/Ago2), a DEAD-box helicase (Gemin-3) and a protein of unknown function (Gemin-4) [15]. Several components, such as the nuclease that cleaves the target, remain to be identified. Introduction of long dsRNAs in mammalian cells triggers a potent interferon response that leads to a general silencing of gene expression by shutting off protein synthesis. This can be circumvented by the use of short synthetic siRNAs made to look like the natural products of the Dicer enzyme. These siRNAs are directly taken up by RISC, and used for silencing without triggering the interferon response.

well as the endogenous gene are silenced. It was later shown that transcripts are produced from both loci, but are quickly degraded in the cytoplasm; hence this phenomenon was termed post-transcriptional gene silencing (PTGS; reviewed in [7,8]). Several observations supported the notion that PTGS involves an RNA intermediate. For example, RNA viruses could induce the silencing of homologous plant genes, and transcriptionally active genes were found to be more effective in inducing silencing than transcriptionally inactive genes. Importantly, plants undergoing PTGS were found to contain RNAs 21–25 nucleotides in length that were complementary to both the sense and antisense strand of the silenced gene [11]. These short RNA species were absent from plants not undergoing PTGS. This hinted at the existence of dsRNA molecules that mediate PTGS, a notion supported further by the finding that transgenes arranged in an inverted repeat are more likely to induce PTGS than transgenes that are integrated as direct repeats [12,13].

Indeed, based on observations made in *C. elegans*, Fire, Mello and colleagues [2] were able to demonstrate that dsRNA is a potent inducer of gene silencing, a process they termed RNAi. This phenomenon appeared to be related to PTGS in plants, as siRNA molecules (21–23 nucleotides in length) similar to those described in plants were found in cells undergoing RNAi [14]. Subsequent work made it clear that RNAi in *C. elegans* also occurs post-transcriptionally, and depends on two important steps (reviewed in [15]). The first step involves the processing of long dsRNA molecules into siRNA, and the second step involves the cleavage of the mRNA that is complementary to the siRNA (Figure 1). It should be noted that siRNA only seems to act on spliced RNA molecules, as it was shown that dsRNA molecules directed against introns are ineffective at inducing gene silencing.

Processing of the long dsRNA molecules into siRNA involves the action of a dsRNA-specific endoribonuclease belonging to the ribonuclease III family of nucleases. This enzyme, known as Dicer, was first isolated from extracts of *Drosophila melanogaster* embryos [16], but was later shown to exist in a large variety of species ranging from fungi to humans ([3,8], and references therein). Throughout these species, Dicer appears to be essential for RNAi induced by dsRNA. Interference with the function of Dicer in *Drosophila* S2 cells results in an impaired ability to silence gene expression by dsRNA [16]. Also, in *C. elegans* a mutant has been identified in the Dicer homologue (DCR-1) that is resistant to gene silencing by dsRNA [17–19].

Following their production by Dicer, the siRNAs are incorporated into the RISC (RNA-induced silencing complex; reviewed in [3]). RISC contains a helicase that can unwind the duplex siRNA, and an endoribonuclease that is distinct from Dicer which is responsible for the cleavage of the target mRNA (Figure 1). In addition, RISC appears to comprise proteins of the Argonaute family, but their function in the complex is currently not entirely clear [20]. RISC uses the sequences of the antisense strand of the unwound siRNA to identify the complementary mRNA, and to promote its specific cleavage. Surprisingly, in *C. elegans* introduction of just a few copies of dsRNA was shown to be sufficient to silence a large excess of target mRNA molecules [2]. This suggested that one molecule of dsRNA can promote multiple cycles of mRNA cleavage, or that the initial signal is somehow amplified. A possible explanation for this amplification came from the finding that, in addition to siRNA encoded by the introduced dsRNA, siRNA molecules were identified that contained sequences that lie outside the original targeting area [21]. This indicated that RNAi can spread to adjacent regions, a phenomenon termed transitive RNAi. The underlying mechanism for transitive RNAi is not exactly understood, but it appears to involve an RdRP (RNA-dependent RNA polymerase) that utilizes the original siRNA as a primer to form *de novo*-synthesized dsRNA that can subsequently be processed by Dicer into secondary siRNAs [21–24]. Consistent with the involvement of an RdRP, RNAi

indeed transits in a unidirectional fashion in worms, so that secondary siRNAs are only produced against sequences positioned 5′ to the original targeting area [21]. In contrast, in plants RNAi can transit in both directions [25]. Although an exact explanation for this bidirectional transitive RNAi is currently lacking, it is clear that the generation of secondary siRNAs directed against sequences downstream of the original targeting area also depends on the presence of an RdRP activity [25]. It should be noted that RNAi amplification appears to be restricted to plants and worms, and is not observed in mammalian cells.

In addition to the amplification of RNAi within one cell, the silencing effect of siRNAs can spread throughout the whole organism in plants and worms [2,26]. Such systemic RNAi requires that a component of the RNAi machinery be passed over the cell membrane to neighbouring cells. The amplification of the RNAi as described above can then ensure efficient gene silencing in the surrounding cells as well. In fact, in worms the silencing effect can even be observed in the progeny of worms treated with dsRNA, a process known as heritable RNAi [2]. However, it should be noted that neither systemic RNAi nor heritable RNAi has been observed in mammalian cells thus far, probably due to lack of an amplification pathway. Systemic and heritable RNAi are both poorly understood processes. In *C. elegans*, genetic screening for mutant worms that lack the systemic response has allowed the identification of SID1 (systemic interference defective 1) [27]. SID1 is a transmembrane protein that may allow the transport of dsRNA or siRNA from cell to cell, thus enabling the spreading of the RNAi response throughout the organism.

As mentioned above, components of the RNAi machinery have been conserved throughout a large variety of species, including vertebrates. However, for several years RNAi approaches were not applicable to mammalian cells because the introduction of dsRNAs of length greater than 30 nucleotides triggered a very strong interferon response, which resulted in non-specific gene silencing due to an overall shutdown of protein synthesis (reviewed in [4]). This impasse was broken when Elbashir et al. [5] and Caplen et al. [6] reported that introduction of synthetic, double-stranded siRNAs of 21–23 nucleotides in length can efficiently and specifically silence gene expression from the complementary gene. These synthetic siRNAs were made to resemble Dicer cleavage products, and are directly incorporated in the mammalian RISC to target mRNA for degradation. This opened the door to RNAi approaches in mammalian cells, albeit that the gene silencing was transient due to the lack of an amplification pathway. Since then, several alternative strategies have been developed, some of which also allow sustained gene silencing (for a review, see [28]). The most widely used approaches rely on stable expression of shRNAs (short hairpin RNAs) from plasmid vectors [29–34]. These vectors are designed to contain an inverted repeat of 19–29 nucleotides encompassing the desired targeting sequence, separated by a short loop of 6–9 nt. Upon synthesis of the nascent RNA, it will form a stem-loop structure that is processed by Dicer to functional siRNAs that can be taken up by RISC and direct sequence-specific mRNA degradation [9]. Commonly, these vectors use pol III (RNA polymerase III) promoters that are normally used by the cell to drive expression of short RNAs, such as tRNAs. pol III has the advantage that transcription starts at very well-defined sequences and lacks a polyadenosine tail. Instead, transcription terminates at a stretch of five thymidine nucleotides, and the nascent RNA is processed in such a way that the 3′-terminus will contain two uridine nucleotides, resembling the ends of natural siRNAs. Vector-driven shRNA turns out to be very effective in gene silencing, and can be used to silence gene expression over long periods of time (reviewed in [28]). In addition, retroviral, adenoviral and lentiviral vectors have been developed that allow RNAi experiments in cells that are notoriously difficult to transfect [35–38]. With these technologies, it is now possible to attain effective gene silencing in transgenic embryos and adult mice [39,40].

Evolution of RNAi as a practical approach in the laboratory has been very rapid, but our understanding of the physiological roles of RNAi has also increased dramatically over the last couple of years. Several lines of evidence support a role for RNAi in a cell-based defence mechanism that protects the genome against mobile genetic elements, such as viruses and transposons (for reviews, see [1,3,7,8]) (Figure 2). Mutant plants that are defective for PTGS are hypersensitive to infection by certain viruses, and the recovery of plants from a viral infection is associated with the selective degradation of viral RNA (reviewed in [41]). *C. elegans* mutants that are defective in RNA silencing display increased rates of transposition [42]. It is assumed that the dsRNA structures that are formed during the replication or transcription of these mobile genetic elements can trigger an RNAi response that can limit their spread. In addition, it has become clear over the last couple of years that RNAi is also used for the regulation of endogenous gene expression during development (reviewed in [43]). Two short RNAs, *lin-4* and *let-7*, have been identified in *C. elegans* that determine timing and sequence of post-embryonic development [44,45]. As a reflection of their important role in the regulation of developmental timing, these RNAs were named stRNAs (small temporal RNAs). The *lin-4* and *let-7* precursor RNAs are approx. 70 nucleotides in length and form a hairpin structure. These hairpins are processed to RNAs that are 22 nucleotides in length, which act as negative regulators of a number of other developmentally important genes, such as *lin-41* (*let-7*) and *lin-14*, *lin-28* (*lin-4*) [44,45]. Thus stRNAs appear to behave in a manner analogous to siRNAs. Indeed, processing of *lin-4* and *let-7* is impaired in *dcr-1* mutants of *C. elegans*, suggesting that stRNAs and siRNAs may be produced and processed by a common pathway [18,19]. However, although both *lin-4* and *let-7* can cause gene silencing, they do so in a manner distinct from siRNA. The *lin-4* RNA only displays partial sequence identity with its target mRNAs *lin-14* and *lin-28* [44], and the same is true for *let-7* and its target *lin-41* [45]. Both *lin-4* and *let-7* bind to a sequence present in the UTR (3′ untranslated region) of their respective target mRNAs through partial base-pairing. This binding results in repression of translation rather than mRNA degradation [44,45], clearly setting the action of stRNAs aside from that of siRNAs. Interestingly, the *let-7* RNA turned out to be remarkably conserved from worms to humans, an observation that led to the proposal that stRNA function is also involved in developmental timing in higher organisms, and that multiple RNAs of similar function might exist [46]. Indeed, hundreds of stRNA-like RNAs have since been identified in humans, mice, fish, plants, flies and worms (for a review, see [43]). In fact, the total number of genes that encode an stRNA-like RNA is estimated to represent approx. 1 % of all genes in these organisms [47–50]. This newly identified class of endogenous non-coding RNAs was termed microRNAs (miRNAs), to set them aside from siRNAs that are derived from dsRNAs produced by viruses, transposons or transgenes. Initially, it was assumed that miRNAs act fundamentally differently from siRNAs, by repressing translation of endogenous mRNAs instead of cleaving them. However, this was shown to be only partially correct, by the demonstration that plant miRNAs that have extensive sequence similarity to their target mRNA can promote their degradation [51–53]. In addition, *let-7* stRNA can promote the cleavage of a target mRNA if it contains a fully complementary target site [54]. Inversely, a siRNA can repress translation of its target mRNA if the sequence similarity is reduced [55]. Thus different classes

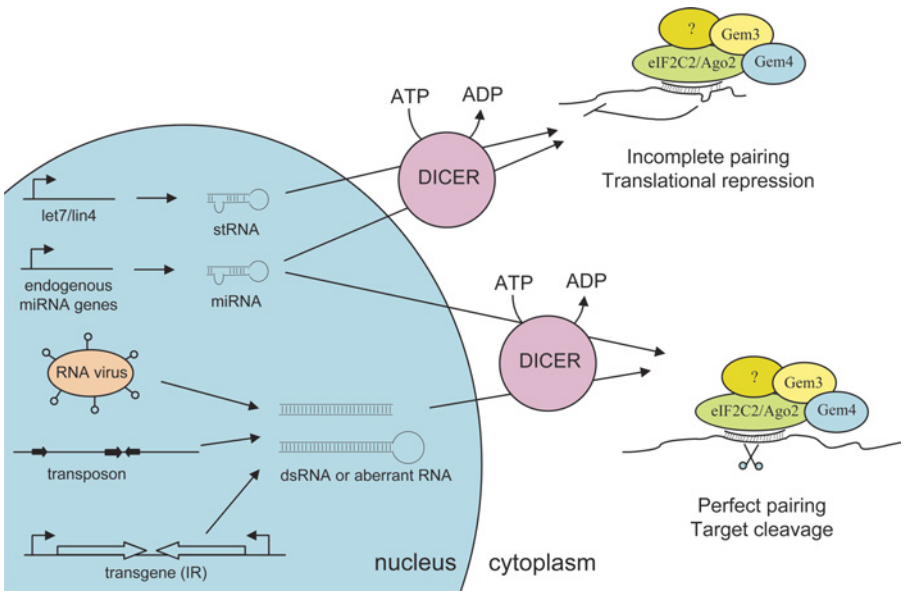

Figure 2 Endogenous RNAi pathways

RNAi is an evolutionary mechanism that is used not only as a cell autonomous antiviral defence mechanism, but is also thought to restrict the spreading of other mobile genetic elements, such as transposons. The dsRNAs that are produced by a replicating virus, or from a retro-element, are transported to the cytoplasm where they can be processed by Dicer to form functional siRNAs. These siRNAs can be used for targeted RNA degradation, or used for RNA-directed DNA methylation (reviewed in [7]). RNAi is also observed when transgenes are introduced into plants, which can lead to the production of aberrant dsRNA molecules. In addition, RNAi is used during development, a mechanism most extensively studied in *C. elegans*. Here, genes such as *let-7* and *lin-4* encode short RNAs that form a $\approx$70 nucleotide hairpin. These hairpins are processed by the Dicer enzyme to form small duplex RNAs of $\approx$22 nucleotides. Owing to their limited, timed expression at specific points during development, these RNAs were termed stRNAs. Later, many more similar RNA species have been identified in multiple organisms, that were subsequently termed miRNAs. It is not unlikely that tens to hundreds of miRNAs are encoded within the genome of higher organisms, and their function may encompass multiple cellular functions.

of interfering RNAs can be discriminated on the basis of their mode of action, mRNA degradation or repression of translation. However, the distinction between the two classes is not a simple reflection of the source from which they are produced, but is rather determined by the sequence similarity between the interfering RNA and its target mRNA.

The finding that partially homologous small RNAs can result in gene silencing is of course disturbing for the design of specific targeting strategies. As a consequence of the reduced stringency for base-pairing between target mRNA and the interfering RNA, it will be more difficult to prevent gene silencing of additional genes besides the intended target. For this reason, it will be essential to delineate the criteria for interaction between target and interfering RNA that are required for efficient translational repression. With this knowledge, one can design more specific targeting vectors that avoid unintentional translational repression of other genes.

APPLYING RNAi IN MAMMALIAN CELLS

Following the initial demonstration that synthetic dsRNAs that resemble naturally produced siRNAs can induce sequence-specific gene silencing after transfection into mammalian cells, many research groups adapted this procedure to study their gene of interest (for examples, see [28]). It is now very apparent that this approach can silence expression of a large variety of proteins, ranging in abundance and stability. As effective as it turned out to be, this approach is limited by the transient nature of the silencing procedure. As the synthetic siRNAs are turned over by the cell, the silenced genes can recover in time, limiting this approach to analysis for periods up to 6 days following a single transfection. On the other hand, the approach takes enormous benefit from the fact that several cell types display very high, if not complete, transfection efficiencies when using synthetic siRNAs. Typically, the transfection efficiencies obtained with synthetic oligonucleotides are well above those reached with plasmid DNA [56]. Such high transfection efficiencies can alleviate the need for selection of the transfected population in order to analyse gene function in a clean background. In addition, copy numbers of siRNAs that are taken up by the cell can be very high, which is an important consideration in mammalian cells, considering that there is no mechanism in place that allows amplification.

As mentioned above, prolonged gene silencing has become possible with the development of expression vectors that allow the production of shRNAs that can be converted by Dicer into functional siRNAs [9,57]. These vector-driven systems for RNAi in mammalian cells now come in many different 'flavours' (Figure 3). Most are based on the production of a single RNA from a pol III-driven plasmid that forms a stem-loop structure in which the sense and antisense strands form the stem of the hairpin [29,31,33,58]. Termination of transcription at a stretch of thymidine bases results in the generation of a 2–4 bp uridine-nucleotide overhang at the 3′ end, identical with the overhang that is normally produced by the Dicer enzyme. Other systems make use of two independent vectors, one driving expression of the sense strand, while the other encodes the antisense strand, both constructed so that they generate the appropriate 2–4 nucleotide overhang [33,59,60]. In an alternative approach, a pol II-driven plasmid was used to produce RNAs modelled after naturally occurring miRNAs, and this turned out to be very effective at accomplishing gene silencing as well [34].

Based on the various strategies, retroviral, adenoviral and lentiviral vectors have been developed that allow introduction of siRNA-encoding vectors at high efficiency in primary cells, as well as in cell types where little or no proliferation is taking place

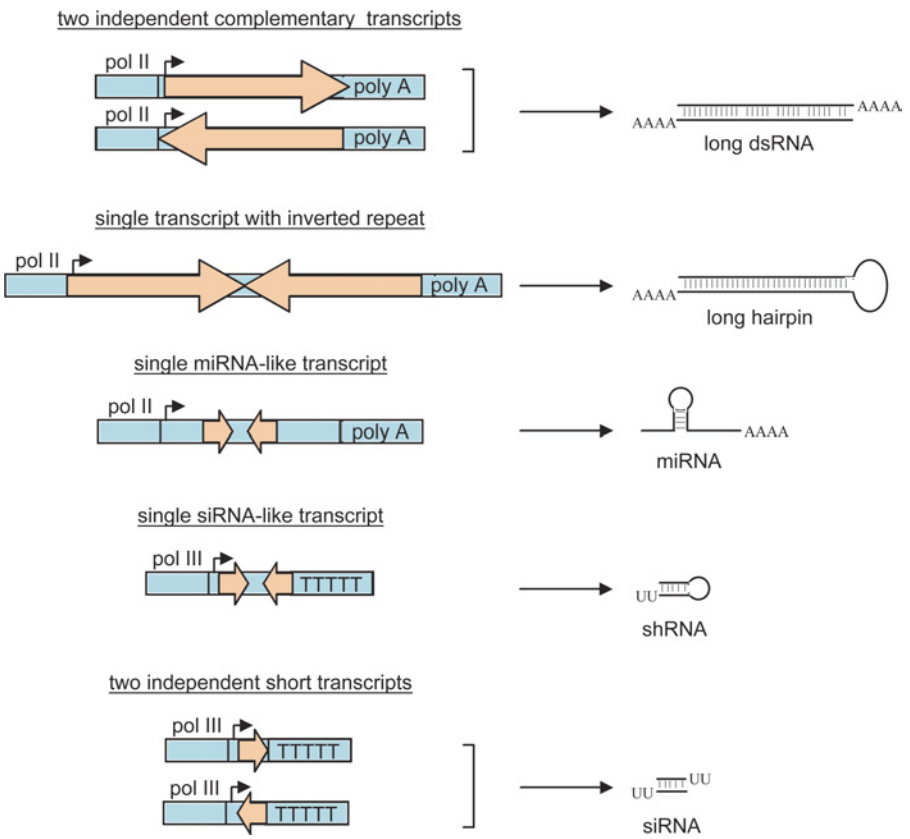

Figure 3 Vector-driven RNAi production

Several strategies have been described for constitutive production of interfering RNA molecules in mammalian cells. First, is expression of long dsRNAs in mammalian cells, either from independent expression constructs or a single construct that contains an inverted repeat. These RNA molecules are typically around 500 nucleotides in length and have to be processed by Dicer in the host to produce siRNAs. Multiple promoters can be used, because the start and termination of transcription is not very critical here. Such systems only work in embryonic cells, or somatic cells that lack the interferon response. A number of systems have been developed to allow long-term RNAi in most mammalian cells. In one, a short RNA was modelled after a naturally occurring miRNA molecule that forms a hairpin [34]. Expression of this miRNA-like molecule was driven from a pol II promoter. Currently the most commonly used systems are modelled after siRNA molecules. For these, RNA pol III promoters are used for their defined start and termination sequences, and because termination generates a stretch of uridine nucleotides causing the ends of the RNA molecules to look identical to natural siRNAs. Sense and antisense strands of the siRNA are either produced from independent expression vectors [59,60], or through expression of shRNAs that are efficiently processed by Dicer [29–32].

[35–38]. Finally, a number of inducible systems for siRNA production have been reported that enable rapid on/off switching of siRNA expression through tetracycline- or ecdysone-responsive transcriptional elements embedded in pol III-specific promoter constructs [61–64]. These methods allow for tightly controlled gene silencing, which is particularly useful for genes that are essential for cell viability. With the establishment of stable clones that contain such inducible vectors, one can work towards a well-controlled, highly reproducible cell system in which the function of a gene can be switched off at will.

With all of these developments, it is clear that our capacities to modulate gene expression in a sequence-specific fashion are rapidly expanding, providing us with excellent opportunities to modulate the activity of a given gene where and when we want. However, the possible approaches one can apply to a given siRNA will be determined in large part by the efficacy of gene silencing that is elicited by that particular siRNA. A major difference between these different targeting strategies is the effective number of siRNAs that will be produced within the cell. The efficacy of synthetic siRNAs is well established, but as discussed above, the copy number of siRNAs in the cell is expected to be very high in this approach. Similarly, transient transfection of siRNA-encoding vectors will result in the production of shRNA in very large quantities that can be processed to siRNAs by Dicer. Nonetheless, shRNAs appear somewhat less effective in mediating gene silencing than synthetic siRNAs [30], and we found examples of target sequences that allow effective knock-down by transfection of synthetic siRNA, while vector-driven shRNAs directed against the identical target sequence were ineffective. Also, the number of siRNAs molecules that is produced in the cell will drop off dramatically upon selection of cell lines that stably produce the siRNA, simply because only one or a few copies of the corresponding vector will stably integrate in the genome of the host. A similar restriction applies to the inducible systems. Nonetheless, these approaches have been shown to work quite effectively if efficient siRNAs are used [61–64]. Thus, in order for these approaches to be successful, one needs to select a target sequence that is recognized very efficiently by the siRNA, so that a minimal number of copies of siRNA is sufficient to effectively shut off protein expression from that particular gene.

SELECTING THE OPTIMAL TARGET SEQUENCE

The first requirement to set up a successful procedure to target a gene of choice is the selection of a good target sequence within that gene. Particularly with synthetic siRNAs, this is a critical issue, considering the costs of a set of oligonucleotides. Unfortunately,

there is not much solid groundwork to rigorously address this issue to help out researchers when selecting a target sequence. Because of this, selecting an effective siRNA is mostly a matter of trial and error, where researchers often have to go through multiple rounds of picking target sites, synthesizing siRNA, and testing their efficacy. Initially, several guidelines were developed for the design of effective siRNAs on the basis of work done with the synthetic siRNAs [5]. The synthetic siRNAs were made to resemble the natural products of Dicer, duplexes of approx. 19 nucleotides with an overhang at both 3′ ends of 2 uridine nucleotides. Thus target sequences of 23 nucleotides were selected that conformed to the consensus sequence of $AAN_{19}TT$, where N can be any nucleotide. The target sequence was selected anywhere between 100 nucleotides downstream from the ATG and the stop codon, to avoid interference by RNA-regulatory proteins that bind the 5′ or 3′ UTR. As a rule of thumb, the GC content had to vary from 30 to 70 %, and a high G content had to be avoided. However, effective siRNAs did not always appear to adhere to these rules. For example, working siRNAs have been described that do not conform to the $AAN_{19}TT$ consensus sequence, as well as siRNAs that target the 3′ UTR [56]. In fact, this latter approach was put forward as a very advantageous one, since it allows simple reconstitution of protein function by co-transfection of cDNAs encoding the targeted gene, but lacking the 3′ UTR. Thus, for some time, it was up to the investigator to select a target site and select the rules to adhere to.

More recently, substantial progress was made with respect to selection of an optimal targeting sequence. This advance came in part from studies that attempted to resolve how the RISC would 'decide' which strand to use from the double-stranded siRNA to direct mRNA degradation [65]. Obviously, the antisense strand of an siRNA needs to be incorporated into the RISC to direct cleavage of the target mRNA, but it was not known whether or how RISC can distinguish between sense and antisense strands for target selection. To address this issue, *in vitro* studies on RISC-mediated cleavage were performed using either single-stranded or double-stranded siRNAs, and the complementary sense and antisense target mRNA [65]. From those studies, it became clear that both strands of a given siRNA could function equally well in directing cleavage of sense or antisense mRNA respectively, provided that they were added as single-stranded siRNA. However, when the siRNAs were added as double-stranded molecules, a significant strand bias was noted. Careful analysis of this strand bias for incorporation into RISC demonstrated that the relative stability of the base-pairs at the respective ends of the duplex determines which strand is taken up by RISC, and directs target selection [65]. In parallel with these studies, others compared sequences of a large group of naturally occurring miRNAs to determine whether certain rules for optimal target sequences could be derived from them [66]. Indeed, these investigators reached similar conclusions; that is, the relative strength of base-pairing needs to be such that the 5′ end of the antisense strand has a lower stability than its 3′ end. Thus the most effective miRNAs/siRNAs would typically have an A–U base-pair at the start of the antisense strand, while the sense strand would have a G–C base-pair at its 5′ end. Moreover, base-pairing was also found to be weaker near the centre of the miRNAs/siRNAs. Clearly, the validity of these observations for mRNA targeting *in vivo* needs to be tested in greater detail, but studies like this do set the stage for rational target site selection and more effective targeting strategies. With all the efforts that are ongoing worldwide to understand and optimize RNAi, it will not be long before we have superior know-how at our disposition that will help us design effective RNAi strategies and reduce much of the frustration encountered in the early days of RNAi.

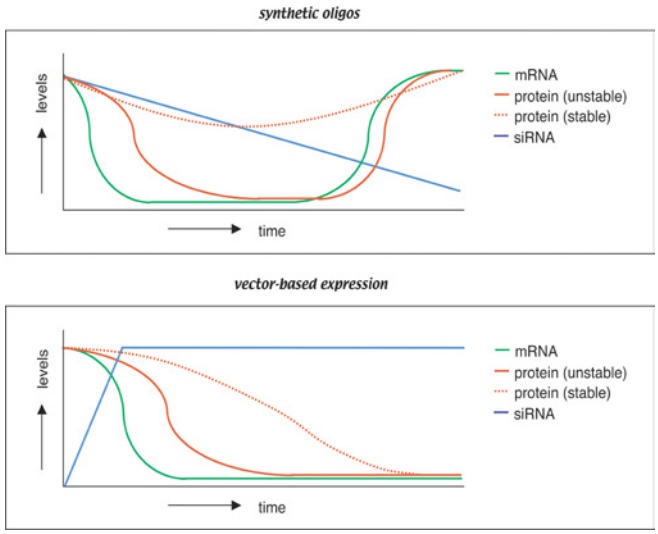

Figure 4 Protein stability and RNAi

Protein stability will affect the time required for effective knock-down of expression below a critical level where the function of that given protein is completely blocked. Using synthetic oligonucleotides that will be turned over by the cell over time, a protein may simply be too stable to obtain such an effective knock-down, although this can eventually be obtained using a system for stable expression of the siRNA. Also, the particular function that is being impaired is an important consideration. Arguably, enzymic functions may still run at very low concentrations, while it may be much easier to remove a function that depends on stoichiometric interactions.

COMMON COMPLICATIONS IN THE USE OF RNAi

The single most important criterion that distinguishes a successful RNAi experiment from a failure is the time required to reduce protein expression below the threshold level that is critical to sustain normal protein function. This is in large part determined by the efficacy of the siRNA to target the mRNA of choice, but, in addition, protein stability is a critical factor. The time required to reduce protein expression below the critical level, once the mRNA is degraded or translation is shut off, is primarily determined by the half-life of that protein. Silencing expression of stable proteins may require very long incubation periods with siRNA that can only be accomplished by stable expression of the siRNA. Longer incubation periods with RNAi increase the chances of secondary effects and adaptation in an ever-increasing fraction of the cells in that population (Figure 4).

Typically, RNAi experiments are performed by transfecting siRNAs into an asynchronous cell population, and the disappearance of the protein under investigation, as well as the appearance of a possible phenotype, are taken as evidence for an effective targeting strategy. However, the outcome of such experiments can be troubled because of many reasons. First of all, it is hard to rule out that the siRNA used strictly targets the selected gene and not some other gene, either by acting as a bona fide siRNA that degrades the mRNA, or as miRNA to block its translation. In fact, it is now clear that subtle mismatches are tolerated in an siRNA, and that partial homology can be sufficient for effective targeting of unintended genes [67–69]. Such mismatches do affect the targeting efficiency, but do not completely abolish the function of the siRNA. Thus it is very likely that additional genes are silenced by a given siRNA. This is commonly controlled for by selecting a second independent siRNA that targets a distinct region in the gene of choice [68,69]. Because the second siRNA is unlikely to target off-targets similar to those of the original siRNA,

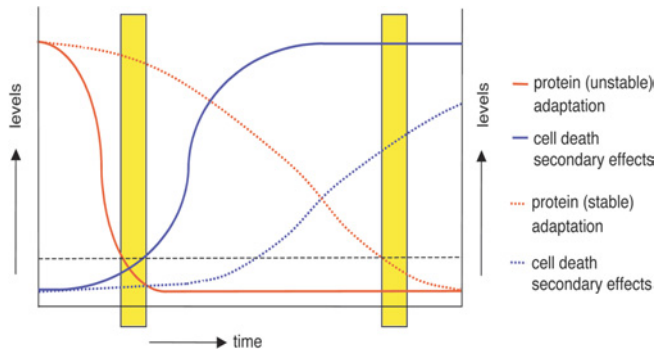

Figure 5 RNAi and secondary effects

The time required to attain a knockdown to levels below the critical protein concentration to retain functionality (shown by the dashed black line) will be different for distinct proteins. This determines a window of opportunity to perform the desired analysis of cell function (shown by the yellow boxes). However, during the depletion secondary effects, such as cell cycle defects, adaptation or even cell death will accumulate in the population. These secondary effects can greatly affect the outcome of an experiment, and will be more troublesome once longer incubation periods are required to remove a given protein function.

this can rule out the explanation that the observed cellular behaviour is due to the silencing of an unexpected off-target.

Another complication that perturbs many siRNA approaches is the fact that secondary effects can arise due to depletion of a specific gene product, which are scored as a primary consequence of impaired gene function. One recently described secondary effect is the activation of an interferon response similar to that described with long dsRNAs [70], but more subtle secondary effects can be invoked as well. Depletion of a gene product by RNAi builds up over time, and it seems likely that some cells in a given population will reach that critical level of protein expression required to impair protein function at a very early stage during the course of an RNAi experiment. In contrast, another fraction of the cells may take much longer to reach that threshold level of protein expression below which gene function is effectively blocked (Figure 5). This complication is aggravated by the variation in siRNA production, or uptake between the different cells in a population. Indeed, siRNA-induced phenotypes often occur in different degrees in mammalian cells, suggesting that the effectiveness of RNAi varies throughout the culture. This can be perceived as an advantage, as it sometimes allows one to study multiple functions of a single gene. However, once a cell lacks a specific gene function, it can quickly degenerate, possibly disturbing the interpretation of the experiment. For example, cells may start dying off if an essential gene is interfered with, or cells in which a given gene is silenced for a considerable amount of time may adapt by increasing expression of related genes. Specifically for experiments that are aimed at understanding the regulation of cell division, complications will arise as cells undergo a failed division after interference with an important cell cycle regulatory protein. This can trigger an indirect checkpoint response, mitotic catastrophe or mitotic failure, so that the depleted cells accumulate all kinds of secondary defects. RNAi effects occur asynchronously over the different cells in a population. As a consequence, secondary effects, adaptation and toxicity will also occur asynchronously, making it difficult to identify the ideal window of opportunity to perform an interpretable RNAi experiment (Figure 5). This becomes increasingly difficult if the time required to reach that critical threshold for protein function increases because of an inefficient targeting strategy, or when a particularly stable protein is studied. Moreover, the expression of the gene under investigation may vary significantly over the different cells in that population to begin with. This is of course particularly true for genes that display a cell cycle-dependent pattern of expression. As mentioned above, RNAi experiments are typically performed by transfection of an asynchronous cell population, and therefore this can be expected to introduce a major variation in timing required to impair gene function from cell to cell in that population. As a simple example of how this can lead to misinterpretation of gene function, an asynchronous population in which a gene is silenced that encodes a protein essential for cytokinesis will accumulate cells with a tetraploid DNA content. If one were to analyse centrosome numbers or mitotic spindles in such a population, one could reach the conclusion that the gene of interest plays an important role in centrosome duplication, since the 'contaminating' tetraploid cells have double the numbers of centrosomes and form multipolar spindles upon entry into the subsequent round of mitosis. But much more subtle secondary effects can be introduced into the culture as well, which can trouble the interpretation of RNAi experiments. For example, one can try to establish the direct involvement of a gene product in a specific signal-transduction pathway. But what if this gene product functions in a second pathway that drives the expression of a gene involved in the signal-transduction pathway under investigation? In an experimental set-up where the secondary effects are allowed to accumulate, it would be very easy to reach the conclusion that the gene of interest is directly involved in the first pathway. Of course, these are well-known complications for genetic approaches, but as many non-geneticists will be taking on RNAi, these are important considerations to make when trying to interpret the outcome of an RNAi experiment.

OPTIMIZING APPLICATION OF siRNA FOR CELL CYCLE ANALYSIS

Asynchronous depletion of an essential cell cycle regulatory protein will progressively affect the cell cycle distribution of the cell population exposed to RNAi. This can have substantial consequences for the behaviour of cells in that population, and as the rate of protein depletion varies from cell to cell, this will certainly affect the interpretation of that experiment. Ideally, one would like to deplete a given protein from a population of cells that does not depend on the function of that protein at the moment of depletion, and subsequently switch to a condition where the function of that protein does become essential. In such an experimental setting, depletion can occur while secondary effects will not accumulate in the population because the cells are not affected by the loss of that protein. Thus, for an ideal RNAi experiment, one would have to think of a situation where the gene of interest does not play a critical role: admittedly, not an easy task, particularly if the function of that gene is unknown. However, for cell cycle-regulated genes the solution to this may be relatively straightforward (Figure 6). For example, a protein that plays a (suspected) role in mitosis can safely be depleted from cells in a G_1 state, provided that the action of this protein is not also required during G_1. Alternatively, a protein that is important for the G_1–S-phase transition can probably be depleted from cells in a quiescent state without resulting in secondary effects or adaptation to protein depletion under those conditions. Obviously, such criteria may apply to many cell cycle regulatory proteins, and depletion of such proteins is best performed on synchronized cultures.

In addition, cell cycle-dependent effects should be considered for other reasons as well; for example, if one would like to study the function of a protein that is essential at some stage both in G_1 as well as in G_2 phases. Depletion of that protein from

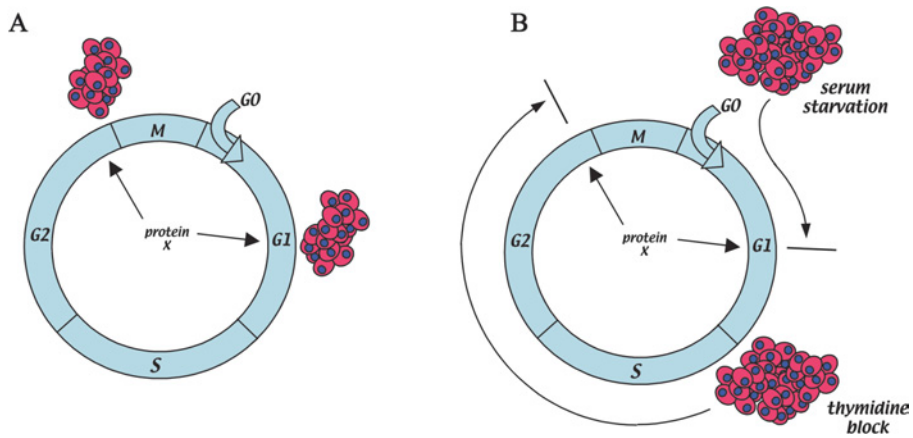

Figure 6 Optimizing siRNA for cell cycle analysis

Removal of a protein that performs multiple essential roles during cell cycle progression will lead to accumulation of the depleted cells at distinct stages in the cell division cycle. If such a population is used for experimentation, then only a fraction of the cells will display the desired effects (**A**). This can be improved by depleting that same protein from synchronized cultures of cells (**B**). This has the additional benefit that secondary effects are mostly prevented from occurring, as the protein can be depleted from a culture that displays no dependence on that particular protein function up until the moment of release from the block.

an asynchronous population of cells would block a fraction of the cells in G_1, while another fraction would arrest in G_2, both effects due to depletion of a single protein (Figure 6A). If one were to try to study the G_1 function of this protein specifically, such an experimental setting would always be troubled by the presence of a large fraction of cells in a state where this particular function cannot be studied. Again, depletion of the protein from synchronized cultures would solve this issue.

Thus, if depletion is performed in synchronized cultures, this allows one to minimize the secondary effects that arise following loss of a specific protein function (Figure 6B). Moreover, upon release of the cells back into the cell cycle, such a synchronous, specifically depleted culture of cells now provides an ideal setting to study the initial defects that occur as a consequence of impaired protein function. This has enormous benefits over the traditional approach of depleting a protein from an asynchronous culture of cells, and analysing that culture at 48 h after depletion. At that point, it is hard to distinguish which of the observed effects are the primary consequence of depletion of that particular protein, and which arose as an indirect consequence.

To provide a clear example of how such an approach can be very useful, it is easiest to provide a working example of an adapted RNAi strategy that utilizes these ideas to circumvent adaptation and secondary effects. When studying mitotic regulatory proteins, we perform protein depletion in a cell population that is blocked at the G_1–S transition [71]. That is, immediately following introduction of the expression vectors encoding the desired shRNAs, cells are incubated with high levels of thymidine that are sufficient to block DNA replication (for a detailed protocol, see [72]). This is a reversible arrest, and these cells can be released from the block after washing out the thymidine. Nonetheless, during this arrest, siRNAs will accumulate and the protein of choice is progressively depleted from cells that have little or no use for this protein at this point in the cell cycle. Concomitantly, this treatment prevents the cells from executing a cell division that could have deleterious consequences and lead to secondary effects, depending on the mitotic function of the protein under investigation. Once the protein is depleted to levels below the critical level for protein function, the cells can now be released and studied as they synchronously move to the point in the cell cycle where the function of this protein becomes critical. In doing this, we can dramatically enrich the cell population under investigation for cells that will display a specific primary defect. This makes the interpretation of the observed effects much more straightforward, and allows a detailed characterization of the exact moment during the cell division cycle where this protein needs to act. It goes without saying that this procedure can be modified to study proteins required at different points in the cell cycle, and indeed others have performed gene silencing in cells forced into a quiescent state by serum deprivation. Obviously, one can take enormous benefit from a setting where depletion is done in such a way that the cells used for the experiment experience little or no defects until the moment one chooses to analyse protein function.

COMPLEMENTATION ANALYSIS

Thus far, most of the applications for RNAi have been restricted to mere RNAi-mediated depletion experiments. However, RNAi does enable us to progress beyond simple depletion and move on to complementation experiments in which mutants of the gene under study are tested for functional reconstitution. For complementation analysis, cells depleted of a given gene product through vector-driven siRNA production are reconstituted with the equivalent gene product, either as the wild-type or in a mutant form (Figure 7). This approach allows one to test the functionality of a given mutant in a cell that is devoid of the corresponding endogenous protein. To accomplish this, one needs to construct expression plasmids encoding the proteins of interest (wild-type or mutant) that can escape down-regulation by the siRNA. For this purpose, we typically introduce a number (>2) of mutations in the cDNA in the sequence recognized by the siRNA [71]. These mutations are chosen in such a way that they will have the most dramatic effect on siRNA/mRNA recognition, without affecting the encoded protein (typically by mutating a G or C at the wobble base position). These silent mutants are then co-transfected with the siRNA, resulting in depletion of the endogenous protein and reconstitution with a mutant of choice.

The first reason to perform such complementation analysis is of course to confirm that a given phenotype is indeed due to depletion of the desired protein, and not a consequence of an off-target. This is an excellent approach to determine the specificity of the targeting strategy. It should be noted that co-transfection with an expression vector containing a cDNA that is not mutated in the

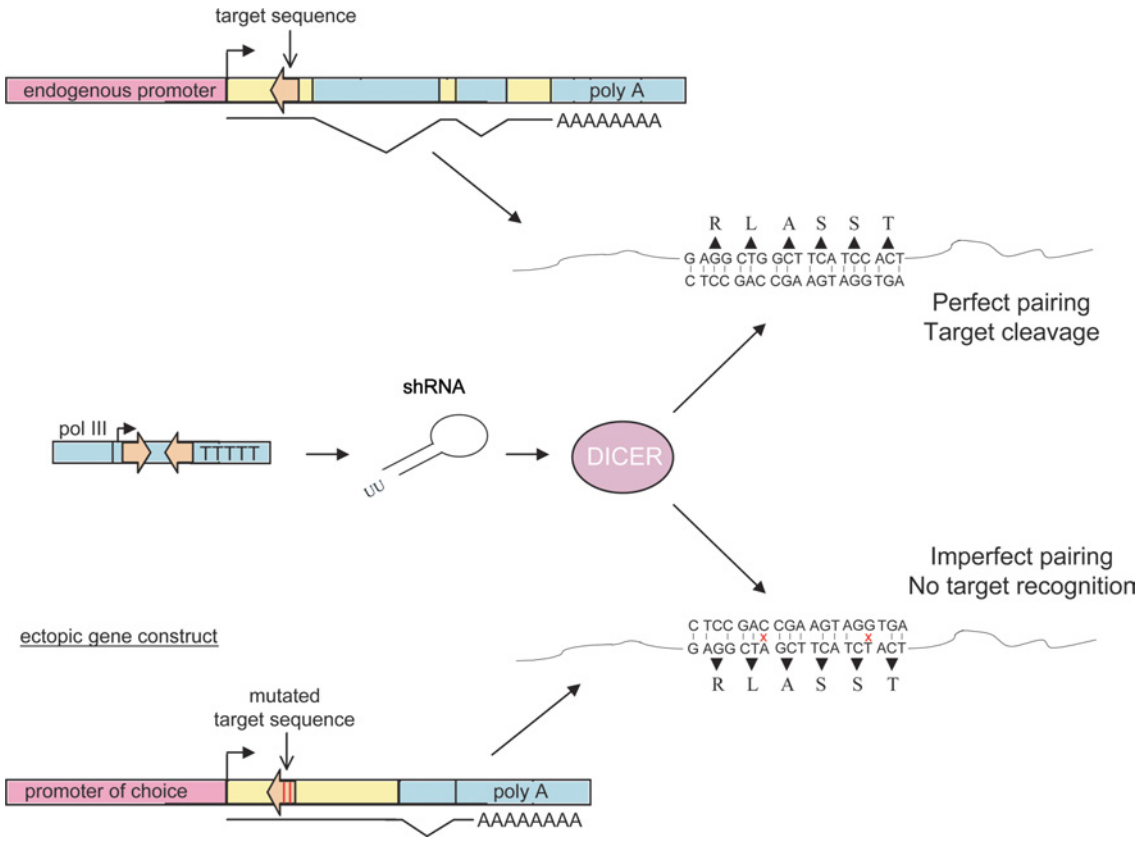

Figure 7 Complementation analysis

By inserting a number of silent mutations in the target sequence of the gene of interest, one can construct a non-silenced variant cDNA that encodes the same protein. The example shown is derived from a working combination of siRNA and non-silenced cDNA [71]. Introduction of an expression construct that contains such a cDNA in combination with the appropriate siRNA now allows one to deplete the endogenous gene product and replace it with the ectopically expressed protein. This protein can either be wild-type to allow one to address the specificity of the targeting procedure, or it can be mutant to allow one to test which functional entities of a protein are required for functional reconstitution. Ectopic expression can be driven from a variety of promoters, ideally from promoters that mirror the periodic expression of the gene of interest by selection either of the promoter of the gene itself, or of another gene that expresses in a similar fashion.

targeting region can also reconstitute protein function, as long as the ratio is such that it can out-compete the produced siRNA. However, the interpretation of this outcome is complicated by the fact that the ectopic mRNA will not only reconstitute protein function, but it will also probably compete for targeting of the additional off-targets. Therefore such an experimental setup can hardly be taken as proof of specificity. In fact, when introducing the construct that contains the mutations at the wobble bases, one should still ascertain that this does not lead to re-expression of the endogenous gene through competition for siRNAs.

The second, equally relevant motivation to set up this approach is that one is now in the position to perform a detailed analysis of functional entities within a protein and test their relevance to the process under investigation. For example, one can now test the importance of single phosphorylation sites on a given protein, analyse functional reconstitution by expression of a mutant protein lacking a specific protein–protein interaction domain, test the requirement of a given kinase domain, and other related issues. Again, several technical issues have to be considered when undertaking this strategy. Expression of the reconstituted gene should not be excessive so as to allow for 'squelching' of binding partners and consequent dominant negative effects. There are several means to accomplish defined expression levels of the reconstituted gene. One can carry out simple titration of the co-transfected plasmid, but reproducibility is a serious problem here. Expression levels are greatly influenced by transfection efficiencies, which are notoriously variable from experiment to experiment. Typically, highly active promoters are used to drive expression of the ectopic genes, which may not allow titration to the levels desired for subtle reconstitution. Moreover, these promoters are highly active at all stages of the cell cycle, and expression of the ectopically introduced gene in phases of the cell cycle where it is normally not present may lead to deleterious effects in the transfected population. Using the actual promoter of the gene studied or a promoter known to mirror cell cycle-dependent transcriptional activity of the endogenous gene can resolve this issue, although the periodic expression from such promoters may require stable integration in the host genome. Thus such promoters may prove ineffective in limiting cell cycle-regulated expression in transient transfections. The solution to this would be to establish stable cell lines expressing the non-targeted mRNA, either from an exogenous promoter or its own promoter. In this way, multiple cell clones can be selected that express the desired protein at levels similar to the endogenous protein. This may seem a very time-consuming approach, particularly if a large collection of mutants must be screened for reconstitution. Nonetheless, this latter approach does have the benefit that reconstitution experiments can be performed in a well-defined system that allows reproducible reconstitution in multiple independent experiments. Moreover, this approach alleviates the need for co-transfection of multiple

plasmids to obtain the knock-down, reconstitution and tagging of the transfected population.

If done properly, reconstitution experiments will prove to be a very powerful tool for mammalian biologists. They allow them to do experiments that were previously only feasible in lower organisms, such as yeast. Clearly, technical issues such as off-target effects, true functional reconstitution and possible dominant negative effects will have to be considered with the necessary amount of caution. However, this technology enables a drastic expansion in the studies we can do in mammalian cells. Previously, we were confined to the analysis of dominant negative mutants that often required high levels of overexpression to compete out the endogenous protein; now, we can study whether a given mutant is functional in the first place, and identify the critical determinants in a protein that are essential for that function.

FUTURE DIRECTIONS

With RNAi we have been handed an enormously powerful tool to study gene function in mammalian cells. Advance in understanding mammalian biology will benefit tremendously from the novel technologies that have been developed over the last couple of years. But at the same time, RNAi will bring us new headaches. Care should be taken not to over-interpret the negative data obtained with RNAi, but temptation to do so will often be hard to resist. Care should be taken to avoid off-target effects, and proper controls, such as a second independent RNAi or complementation with a non-targeted cDNA, should be included. But most of all, the speed at which one can knock down expression of a single protein should not be taken as evidence that this procedure will circumvent any secondary effects. Even when studying mammalian cell division, one should realize that 2 days is twice a lifetime for most of the cells that we study. Thus we should not only marvel at all of the experiments we can now do in our lifetime, but try to devise experiments we can do within a cell's lifetime.

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