Silence of the strands: RNA interference in eukaryotic pathogens

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Double-stranded (ds) RNA interference (RNAi) is a recent technological advance that enables researchers to reduce gene expression at the post-transcriptional level. This form of RNA silencing is initiated by dsRNA, expressed in or introduced into a cell of interest, which triggers homology-dependent degradation of the corresponding mRNA. This versatile technique has remarkable promise as a tool for the study of eukaryotic pathogens. Protozoan parasites and pathogenic fungi often resist manipulation using standard molecular genetic approaches. Researchers studying these organisms need flexible molecular tools, particularly to exploit newly sequenced genomes; this review offers a practical guide to establishing RNAi in pathogenic eukaryotes.

Eukaryotic microorganisms have gained prominence in recent years as significant causes of disease and intriguing subjects for basic research. In this review we consider protozoan parasites and fungi together, because studies of these single-celled pathogens share several features*. Historically, these organisms were selected as research subjects for their inherent interest and medical importance, rather than for their experimental tractability, leading to common technical obstacles. As both groups are pathogens, parallel questions arise relating to how they interact with their hosts. Also, because these eukaryotes share biological features with their hosts, the challenge of developing selectively toxic chemotherapeutic agents to combat the diseases they cause is increased. Finally, study of these organisms is at a similar level of sophistication in many respects: although some molecular manipulations are possible, and new techniques are evolving, these systems lack the broad range of tools available for model organisms; genomes are just beginning to be sequenced; and understanding of unique biochemical and cell biological processes is patchy. Along with the challenges of studying them, however, the parasitic protozoa and pathogenic fungi offer exciting opportunities for basic research and development of therapies. Moreover, their biology has frequently led to fundamental discoveries that also apply to higher eukaryotes.

In the search for molecular tools to aid the study of eukaryotic pathogens, the recently developed approach of double-stranded (ds) RNA interference (RNAi) has enormous potential. RNAi refers to the experimental introduction of dsRNA into a system to specifically target the corresponding mRNA for destruction, thereby reducing expression of the gene product. In the past few years this powerful technique has swept through a broad range of experimental communities, and has now been added to the standard armamentarium for reverse genetic experiments. The goal of this review is to provide researchers examining eukaryotic pathogens with enough background, examples and practical information to apply this method to their microorganism of choice.

Basics of RNAi

RNA silencing is an evolutionarily conserved mechanism that protects genomes from exogenous (viral) and endogenous (transposon) threats, and participates in cellular programs of gene expression and development [1]. This group of processes encompasses RNAi as well as post-transcriptional gene silencing and co-suppression in plants, quelling in fungi and algae, and antisense suppression. Although these silencing mechanisms share components, they vary among species and are triggered by distinct molecular events. RNAi is triggered by the introduction into a cell of dsRNA homologous to a gene targeted for silencing. Genetic and biochemical studies have shown that this dsRNA is first processed into 21–25 nt guide sequences, in an ATP-requiring step, by a dsRNA-specific endonuclease termed DICER (Fig. 1). The resulting dsRNA products, known as small interfering RNAs (siRNAs), associate with a nuclease complex, the RNA-induced silencing complex (RISC) [2], and become unwound. They are then used by the RISC as a guide for homology-dependent degradation of the target mRNA (Fig. 1).

A notable aspect of RNAi is that in many organisms the effects are amplified and sustained after the initial introduction of dsRNA. RNAi amplification could occur through chopping of the introduced long dsRNA into multiple siRNAs, or through reuse of siRNAs by the effector mechanism [3]. Target-dependent amplification [4] could also produce additional dsRNA triggers. This process has been suggested by the involvement of an RNA-dependent RNA polymerase (RdRP) in RNAi in some organisms. It is believed that the RdRP uses antisense
siRNA to prime the conversion of cellular mRNA into dsRNA, thus amplifying interference (Fig. 1) [5]. Interestingly, RdRPs are present and required for RNAi in Caenorhabditis elegans, plants and Neurospora, but do not seem to play a role in humans or Drosophila [6]. Perhaps consistent with this, siRNA-mediated RNAi in human cells is transient [2], in contrast to most organisms in which this process has been studied. The importance of an RdRP in RNAi in only a subset of organisms is an example of the biological variation in this process, and highlights the fact that assumptions should not be made about its effects in different organisms.

What is the natural function of RNA silencing? To date, it has been implicated in defense of the genome through suppression of both transposon mobility and accumulation of repetitive DNA in the germ line [4,6]. In trypanosomes, 24–26-nt siRNAs have been detected that correspond to two retrotransposons, leading to the speculation that RNAi is active in organisms whose genomes contain these potentially destructive elements [7]. The DICER protein has also been linked to developmental events in C. elegans [8], and recent studies suggest that RNA silencing machinery affects gene expression at the level of chromatin structure [9,10]. The combination of exquisite specificity and potent action has made RNAi broadly useful in systems ranging from early work in C. elegans and trypanosomes to more recent studies in mammalian cells. The utility of this technique has prompted development of RNAi methodology in numerous organisms, including several eukaryotic pathogens.

### Applications of RNAi

The great value of RNAi to experimental biologists is its ability to provide information about gene function relatively quickly and easily. Two situations can benefit from this approach to the study of eukaryotic pathogens. The first is when a researcher wishes to analyze a specific gene or gene family in a particular microorganism. This is difficult in many parasites and fungi because efficient methods for gene disruption are lacking. In the case of related genes, duplicated genes or a gene family, these problems are amplified. Classical genetic approaches used in other organisms are often inaccessible because the sexual cycle has not been identified, is developmentally complex or is experimentally unwieldy.

RNAi has many applications in characterizing the functions of individual genes. Regulated expression of RNAi triggers, and inherent variation in the efficacy of interference (see below), allows researchers to examine phenotypes resulting from a range of levels of gene expression. Several unrelated genes can be targeted with little additional effort, allowing epistasis analysis and investigations of multiple processes. RNAi can produce cells with reduced expression of essential genes, yielding phenotypes which could not be studied by a traditional disruption approach. Additionally, the effects of RNAi can be reversible, permitting in-depth study of gene function. In organisms where gene disruption is often laborious or difficult and tools for regulated expression are limited, this is extremely attractive.

Because RNAi is an homology-based silencing system, it can also be used at the level of gene families. Simultaneous interference with homologous family members using a single dsRNA has been demonstrated in trypanosomes [11]. In Drosophila, this has also been achieved using a combination of dsRNAs in the same experiment [12]. Although such interference experiments must be interpreted with care, this approach is significantly more efficient than constructing multiple knockout strains in most protozoan parasites and fungi. In Drosophila and in human cells in culture, RNAi has also been used to selectively degrade specific alternatively spliced mRNA isoforms [13], another exciting application of this technique.

A second experimental challenge is how best to exploit the enormous quantity of sequence data now being generated from many fungi and protozoan parasites (Box 1). In many large-scale projects, RNAi is the approach of choice for genome-wide screens of gene function. RNAi libraries are being used to study Trypanosoma brucei, an approach that could also be powerful in other systems. In C. elegans, researchers are systematically studying the entire genome using RNAi [14], aided by automated phenotype screening. Astoundingly, almost all of the worm’s predicted 19 000 genes have now been analyzed, and similar strategies are being applied to plants.
Drosophila, trypanosomes and humans [9]. Although genome sequencing projects in most eukaryotic pathogens are still at a relatively early stage, these large-scale applications of RNAi should be considered for the future.

RNAi in eukaryotic pathogens to date

*T. brucei*, the causative agent of African sleeping sickness, is the most ancient eukaryote in which RNAi has been demonstrated. Genetic manipulation is difficult in *T. brucei* because it is diploid throughout its life cycle, lacks an easily manipulated sexual cycle, and has many genes in multiple copies. These barriers are largely overcome by the application of RNAi, which has proven extremely useful for studies of trypanosome biology and of the interference process itself [15].

Over the past four years, RNAi studies in trypanosomes have evolved from establishing efficacy to developing a fast, simple and inducible process with stable results in both bloodstream and procyclic forms [16]. RNAi was originally demonstrated using the introduction of synthetic dsRNA [17] by electroporation (Fig. 2a). Later, the adaptation of methods for using a tetracycline-inducible promoter in trypanosomes [18–20] led to the development of inducible constructs for dsRNA expression that allow stable maintenance of the interference effect. Stem-loop constructs (Fig. 2c) are particularly convenient because expression can be assessed by measuring transcription of the loop sequence. Co-transfection with separate plasmids containing sense and antisense fragments (Fig. 2e) also successfully triggers RNAi in trypanosomes [20], as do opposing T7 promoter constructs (Fig. 2d), both episomal and integrated [19,20]. These opposing promoter constructs often generate some dsRNA even in the absence of the tetracycline inducer, but are advantageous because they require only one-step cloning. It is notable that genes vary in their sensitivity to RNAi, as shown by several genes which have not yielded expected phenotypes or demonstrated significant mRNA reduction [19]. Additionally, in all inducible systems for RNAi in trypanosomes, residual mRNA of the target gene is frequently observed, making absolute ‘null’ strains impossible [17,21]. In some cases, a null phenotype is produced despite this residual mRNA, probably depending on the threshold of mRNA required for function of the product.

The first demonstration of RNAi in trypanosomes was the inhibition of *α*-tubulin reported in 1998 by Ullu and co-workers [17] (Fig. 3). Since then, RNAi has been used to study numerous aspects of trypanosome biology, including flagellum ontogeny [22], a mitochondrial RNA polymerase [23] and enzyme compartmentation in glycosomes [24]. Essential genes such as that encoding topoisomerase II [25] and a gene required for flagellum attachment [26] have been studied by observing progress to cell death after RNAi induction. RNAi has also been used to study gene families, such as *TbPDE2C*, a cAMP-specific phosphodiesterase, and the *TbPDE2* family [11]. In that study, researchers used various dsRNA triggers to examine the effects of inhibiting expression of individual family members as well as all family members simultaneously.

**Box 1. Genome project web sites for selected eukaryotic pathogens**

<table>
<thead>
<tr>
<th>Protozoan parasites</th>
<th>Fungi</th>
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<tbody>
<tr>
<td>Leishmania major</td>
<td><em>Aspergillus fumigatus</em></td>
</tr>
<tr>
<td><em>Plasmodium falciparum</em></td>
<td><a href="http://www.aspergillus.man.ac.uk">http://www.aspergillus.man.ac.uk</a></td>
</tr>
<tr>
<td>Toxoplasma gondii</td>
<td><a href="http://www.sanger.ac.uk/Projects/A_fumigatus">http://www.sanger.ac.uk/Projects/A_fumigatus</a></td>
</tr>
<tr>
<td>Trypanosoma brucei</td>
<td>Cryptococcus neoformans</td>
</tr>
</tbody>
</table>

http://cneo.genetics.duke.edu/
Although inhibition of individual genes occurred with varying efficiencies, limiting analysis of the precise role of each enzyme, this study represents a powerful application of RNAi. Finally, trypanosome genomic libraries have recently been constructed in RNAi vectors, allowing forward genetic applications of this technique [27]. The tremendous success of RNAi experiments in trypanosomes inspires optimism that this method will advance studies in other eukaryotic pathogens.

**Plasmodium falciparum** is the dominant species causing human malaria, the most common and deadly disease caused by a protozoan parasite. In contrast to the extensive experience in trypanosomes, application of RNAi to this organism is still at an early stage. Several initial studies reported successful growth inhibition after electroporation of dsRNA into *P. falciparum*, noting either protein reduction, in the case of a serine/threonine protein phosphatase (*P/PPl*) [28], or reduction in mRNA corresponding to a dihydroorotate dehydrogenase (*DHODH*) [29]. An in-depth study was recently published, in which inhibition of cysteine proteases important for hemoglobin degradation was induced by adding dsRNA to the growth medium of erythrocytes harboring this intracellular parasite [30]. The authors demonstrated reduction of the targeted mRNA, production of siRNAs from introduced dsRNA, and concomitant changes in parasite morphology and hemoglobin accumulation consistent with protease inhibition. They also showed that a parasite lysate degraded radiolabeled mRNA in *vitro* only when the original cultures had first been treated with the corresponding dsRNA. This work, along with the earlier results, shows that RNAi functions in *P. falciparum*, and is promising for studies of this parasite.

Two other parasites in which RNAi has been attempted are *Toxoplasma gondii* and *Leishmania major*. *Toxoplasma* is an intracellular protozoan parasite responsible for both disease in immunocompromised individuals and birth defects. The sexual cycle of this organism occurs only in cats and is not easy to manipulate. *Leishmania*, which causes both mucocutaneous and visceral disease, is also intracellular in the host, and its diploid state adds to the difficulty of genetic manipulation. Despite efforts in several labs to establish RNAi in these two important organisms, no success has been achieved to date (S. Beverley, D. Roos, and D. Sibley, pers. commun.). It is possible that the RNAi machinery does not exist in these parasites, and it is interesting to note that *Leishmania* has little interspersed repetitive DNA in its genome, with nothing that appears clearly related to known families of mobile elements (S. Beverley, pers. commun.). Despite these negative results, attempts with different approaches might eventually be successful, especially as we continue to learn more about the mechanisms of RNAi. Meanwhile, work to test RNAi is ongoing in *T. cruzi* and *Entamoeba histolytica* – and probably other protozoan parasites by the time this review is published.

There is great interest in applying RNAi to pathogenic fungi because other RNA silencing processes are well described in their non-pathogenic counterparts. For example, quelling in *Neurospora* provided much of the early data on RNA silencing [31]. Additionally, antisense interference has been used in both pathogenic and non-pathogenic fungi including *Neurospora crassa* [32], *Cryptococcus neoformans* [33] and *Candida albicans* [34]. Because RNA silencing mechanisms share common functions and machinery, it is likely that RNAi will be broadly useful in pathogenic fungi as well.

So far in the fungi, RNAi has only been demonstrated in *C. neoformans*. *C. neoformans* is an encapsulated pathogenic fungus that causes a potentially fatal meningoencephalitis in immunocompromised patients. In this organism, RNAi was induced using episomal expression of a stem-loop construct to target an auxotrophic marker and a gene involved in capsule synthesis (Fig. 4), both individually and simultaneously from a tandem construct [35]. The tandem construct used the marker gene to signal successful interference; this approach facilitates studies where phenotypic outcome is uncertain [35]. RNAi in *C. neoformans* has also been successfully triggered using a non-integrated opposing promoter construct under galactose regulation (I. Bose et al., unpublished).

Efforts are currently being made to establish RNAi in other fungal pathogens such as *Aspergillus fumigatus*, a filamentous fungus responsible for severe disease in immunocompromised individuals, and *Histoplasma capsulatum*, a prevalent dimorphic fungus. In the near future, we anticipate reports of successful use of RNAi in these, and possibly other, fungal organisms.

**Practical considerations**

Establishing RNAi in a favorite pathogen requires a robust method for generating the trigger, a well-chosen target gene, and plans for monitoring mRNA levels and the presence of the gene product. Suggestions for these are detailed below. An additional preliminary step could be to search the organism’s genome (if available) for homologs of genes known to be involved in RNAi, such as DICER or RdRP. While lack of obvious homologs should not preclude testing RNAi, as this is even the case for DICER in *T. brucei*, their presence might energize efforts to establish the method experimentally.

The first experimental step in attempting RNAi in a new organism is establishing a way to introduce dsRNA...
into the cells (Fig. 2). Initial trials can be attempted by simply introducing in vitro-synthesized dsRNA or siRNA (discussed later) [36]. In many organisms, however, use of a construct to produce dsRNA in the target cell has been most effective for sustained interference. Such experiments will be most straightforward with a construct whose expression is readily detectable, which contains a strong promoter, and which interferes with a gene whose downregulation rapidly yields a striking phenotype [21]. The specifics of construct design, and whether the construct is episomal or integrated, will depend on available methods for the organism under study. When designing a construct for production of dsRNA, cDNA sequences are the templates of choice; exon-rich genomic sequences can also be used, but they might not work quite as well [37]. It is not necessary to have the whole gene sequence, an advantage where sequence information is limited. Sequence dependence is strict, however, so caution is required when working with EST or single-pass sequence, as the quality of the sequence will affect the quality of the results [38]. Success has been achieved with sequences from 21 bp to >1100 bp in length, but has not been addressed systematically in most cases. If the stem-loop or opposing promoter constructs present technical difficulties, introducing separate plasmids that encode sense and antisense fragments of the target gene could be an alternative for generating dsRNA in cells [39].

Another approach for performing RNAi is the direct introduction of siRNAs [38]. This avoids triggering a non-specific degradative mechanism that confounds RNAi in mammals [40], and presumably circumvents the need for a functional DICER enzyme. This method is effective in Drosophila and human cells, and can be useful in systems where dsRNA expression is technically difficult [38]. A thorough examination of using siRNAs to trigger RNAi can be found in [41], including commercial sources of siRNAs and suggestions on selection of siRNA sequences. It should be remembered, however, that specifics of preferred siRNA size and character probably vary with the organism.

Once dsRNA corresponding to a gene of choice is introduced into the target organism, the RNAi transformants should be examined for construct expression (where applicable), levels of targeted mRNA, and alteration in levels of the gene product or its putative function [41]. Specific RT–PCR or northern blotting can most readily assess construct expression; in stem-loop constructs, the loop sequence is a convenient marker. mRNA levels can similarly be examined by quantitative blotting or RT–PCR. For detailed mechanistic studies of cells into which dsRNA has been introduced, their content of siRNAs can also be evaluated [7]. Downregulation of gene products can be assessed by examining phenotype or by detection of protein by antibody, activity, or other methods. When analyzing results, it is important to consider that the half-life of the targeted gene product, its regulation, and its concentration in the cell will alter the outcome of RNAi experiments. Also, it could be important to clone RNAi-expressing cell lines before analysis, because substantial variability in the effects of RNAi have been demonstrated, even within the same experiment [35,39]. It is advisable to analyze several independent clones for each experiment, using phenotype or mRNA quantitation to identify strains in which interference is most robust. Use of a marker in tandem, as described above for C. neoformans, can also signal clones of greatest interest. It is possible that RNAi might lead to partial or even no reduction of function for a subset of genes [37]. In some cases, perhaps for mechanistic reasons, RNAi does not accurately phenocopy the null mutant of genes, as with genes involved in neuronal
function in C. elegans [37]. This variability in the effectiveness of RNAi is an especially important consideration when analyzing RNAi libraries.

It is crucial to remember that the observed effects of RNAi could result from interference with genes other than the one for which dsRNA was originally intended. This can occur through two mechanisms. First, as RNAi relies on homology for degradation of mRNA, genes with high levels of homology, such as members of a single gene family or alternatively spliced gene products, can be affected simultaneously by a single dsRNA sequence. Although this can be useful experimentally, as mentioned earlier, it can also complicate interpretation of experiments. Where RNAi is performed on intracellular pathogens there is a chance of interfering with homologous host genes as well, depending on the mode of nucleic acid delivery. The involvement of an RdRP in some organisms can be a second source of confusion because it can extend dsRNA synthesis from the antisense primer into regions upstream of the originally targeted gene (Fig. 1, bottom right). The resulting dsRNA, when processed into second-generation siRNAs, can cause a transitive RNAi effect on genes 5’ to the original target [42]. This effect varies in different systems [5], but because it can yield confusing or multiple RNAi phenotypes it necessitates consideration of the location of the target gene within the genome and can limit the usefulness of RNAi in some systems [13]. In some cases, the presence of genes with redundant functions might mask the effect of RNAi [37], however, because RNAi makes interference with multiple genes easier, it can also be used to address such obstacles. Where possible, exogenous complementation of phenotypes induced by RNAi can prove the link between the intended target and the outcome [19].

The future of RNAi

RNAi represents an exciting breakthrough in the quest to inhibit gene expression on the level of individual genes, gene families and entire genomes. In a short time it has revolutionized studies in systems ranging from worms to trypanosomes. Currently, it allows the exploration of the genomes and biology of numerous organisms, with enormous potential for use in experimentally difficult systems such as eukaryotic pathogens. Other exciting possibilities are on the horizon in the therapeutic arena. RNAi is both effective and stable in mammalian cells through the expression of micro stem-loop structures that produce siRNA-sized dsRNA [43,44], and it is possible that this technology could also be used in whole animals. There is ‘no conceptual barrier to incorporating this strategy for targeted suppression into virus-based delivery vehicles’, for example, to silence an activated oncogene specifically [9]. RNAi has also recently been shown to block viral infection in human cells [45–48]. It could ultimately be useful in additional hosts and against a variety of pathogens, although factors determining success will include identification of appropriate targets, the available delivery systems for the dsRNA, and where the pathogen resides within the host or disease vector. The ability to destroy mRNA based on sequence homology provides the specificity required to destroy invaders while leaving the host unharmed. Application of RNAi to important eukaryotic pathogens will help advance knowledge of their basic biology and potentially yield antimicrobial therapies.

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