

Review

RNA interference: a pathway to drug target identification and validation in trypanosome

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RNA interference (RNAi) is a phenomenon through which double stranded RNA (dsRNA) induces potent sequence-specific degradation of homologous transcripts. Besides its function in cellular defense and developmental regulation, it has emerged as an invaluable tool for elucidation of gene function and drug target validation. This is particularly useful when substantial genome sequence data are available. Gene silencing using RNAi can aid translation of raw genomic sequence data into biologically relevant information toward the development of new and/or improved control strategies. Here, we review the current status of RNAi in trypanosome research focusing on challenges involved in the utilization of the technique as well as its potential application in drug target discovery and validation.

Key words: RNA interference, trypanosomes, gene silencing, RNAi library, drug targets

INTRODUCTION

RNA interference (RNAi) is the phenomenon where double stranded (ds) RNA induces sequence-specific silencing of genes by degradation of complementary RNA in a cell. This silencing phenomenon is widespread in eukaryotes and has been previously linked to post-transcriptional gene silencing (PTGS) in plants (Jorgensen, 1990; Napoli et al., 1990; Flavell, 1994; Matzke et al., 2000; Baulcombe, 2004) and quelling in fungi (Romano and Macino, 1992; Fagard et al., 2000; Vaucheret et al., 2001). It has also been reported in *Caenorhabditis elegans* (Fire et al., 1998), mammalian cells (Montgomery et al., 1998; Elbashir et al., 2001a) and insects (Caplen et al., 2001). It is thought to be an evolutionarily conserved mechanism that inhibits the replication and expression of selfish DNA elements and viruses (Caplen et al., 2001; Chi et al., 2003; Matzke et al., 2002) and hence maintains genome integrity. Although its protective role against pathogens would be very important in plants and worms such as *Caenorhabditis elegans* (Milhavet et al., 2003) which lack humoral defense system analogous to that found in animals, its role in mammalian cells remains unclear. It is suggested that it must have evolved

as a cellular defense mechanism against foreign DNA and RNA regulating the expression of endogenous genes (Milhavet et al., 2003). In addition, the process has been implicated in cellular processes such as gene expression (lorio et al., 2005), differentiation (Song and Tuan, 2006; Callis et al., 2007), proliferation, apoptosis (Jovanovic and Hengartner, 2006), metabolism and signaling (Yoo and Greenwald, 2005) hence involved in animal and plant development (reviewed by Carrington and Ambros, 2003; Harfe, 2005; Kloosterman and Plasterk, 2006).

The biological role of RNAi in disease causing trypanosomes is not clear. Although abolition of RNAi by deletion of genes responsible of the process cause a transient defect in chromosome segregation and mitosis (Durand-Dubief and Bastin, 2003) and augment levels of retroposon transcripts (Shi et al., 2004), parasites lacking RNAi are fully capable of undergoing a complete life cycle (Janzen et al., 2006). The discovery of RNAi in trypanosomes has provided a basis for the elaboration of powerful research tools in reverse genetics studies and utilization of raw and rapidly accumulating information available in the trypanosome genome database. The technique is of immense power in functional genomics. Its ability to specifically ablate the function of individual genes makes RNAi an invaluable tool in drug target discovery and validation. In this review, advances and chal-

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Trypanosome RNAi Vector Systems and RNAi Mechanism

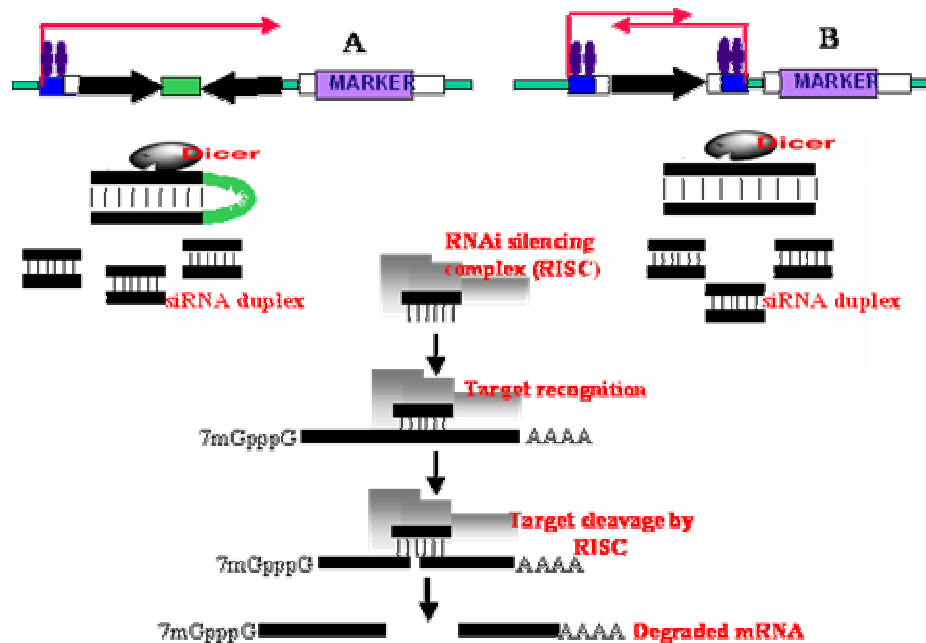


Figure 1. The two types of trypanosome double stranded RNA expression constructs. A: single inducible promoter driving head to head arranged gene fragments. B: two opposing inducible promoters controlling the expression of a single copy gene fragment. Double stranded RNA is cleaved by dicer into 21- to 23-nt siRNAs, which is complexed with a RNA silencing complex, the RISC. RISC unwinds the siRNA to help target the appropriate mRNA. The siRNA-mRNA hybrid is cleaved, releasing the siRNA, and the mRNA is degraded by endo and exonucleases.

allenges of RNAi in trypanosome research with respect to drug target validation are discussed.

RNAi mechanism and classes of small RNAs

Studies over the last several years have demonstrated that RNAi is mediated by the generation of 21- to 23-nucleotide dsRNA molecules, termed small interfering RNA (siRNA) (Elbashir et al., 2001a; Dillin, 2003; Dykxhoorn et al., 2003). They have a characteristic structure, with 5'-phosphate/3'-hydroxyl ends and a 2-base 3' overhang on each strand of the duplex (Dykxhoorn et al., 2003). Upon entry into the cell, dsRNA is cleaved into siRNAs by the action of RNase-III-type enzyme Dicer (Jones et al., 2004; Pauls and Esté, 2004). The siRNAs are incorporated into a protein-RNA complex, the RNA-induced silencing complex (RISC) (Nykanen et al., 2001; Schwarz et al., 2002; Schwarz et al., 2003; Dykxhoorn et al., 2003). There is a strict requirement for the 3' siRNA to be 5' phosphorylated to enter into RISC, and siRNAs that lack a 5' phosphate are rapidly phosphorylated by an endogenous kinase (Dykxhoorn et al., 2003). The duplex siRNA is unwound,

leaving the antisense strand to guide RISC to the homologous target mRNA for endonucleolytic cleavage (Pauls and Esté, 2004). The target mRNA is cleaved at a single site in the centre of the duplex region between the guide siRNA and the target mRNA, 10-nt from the 5' end of the siRNA. Mismatches >1-2bp within the 21- to 23-nt siRNA effectively disrupts proper degradation of the target mRNA (Dillin, 2003). The synthesis of the protein encoded by the mRNA targeted by the siRNAs is thus prevented and that protein is selectively depleted from the cell. The RNAi process is shown in Figure 1.

Presently, four major classes of small RNAs that mediate RNA silencing have been described. They include small interfering RNAs (siRNAs) (Elbashir et al., 2001a; Elbashir et al., 2001b), microRNAs (miRNAs) (Nelson et al., 2001; Lagos-Quintana et al., 2001; Lau et al., 2001), small heterochromatic RNAs (Reinhart et al., 2002) and Piwi-interacting RNA (piwiRNAs) (Aravin et al., 2006; Lau et al., 2006; Brower-Toland et al., 2007). Small interfering RNAs (siRNAs) are 21-23 nucleotide dsRNAs and are the hallmark of 'classical' RNAi. MicroRNAs are processed by the RNase III enzyme Droscha to produce a hairpin RNA of ~70 nucleotides (Lee et al., 2002). The hairpin is subsequently exported into the cytoplasm by

exportin-5 (Jones et al., 2004). Pre - miRNA is then cleaved by the Dicer to produce a double stranded 21- to 23- nucleotide RNA duplex (Grishok et al., 2001; Hutvagner et al., 2001). Like siRNAs, they direct silencing as in siRNA-mediated mRNA cleavage in case of complete complementarity to the target transcript (Ambros et al., 2003; Doench et al., 2003; Chen et al., 2004). In case of incomplete complementarity, miRNA blocks mRNA translation through imperfect complementary binding of the antisense sequence to the miRNA recognition elements (MREs) within the 3' untranslated region (UTR) (Jones et al., 2004). This miRNA translation repression process is distinct from siRNA-directed mRNA cleavage. Doench et al., (2003) have however shown that siRNAs can mediate miRNA-dependent translation repression. In eukaryotes, centromeric silencing has been reported to occur via small heterochromatin RNAs (Volpe et al., 2002). In *Schizosaccharomyces pombe*, they have been implicated in epigenetic modification (Reinhart et al., 2002; Volper et al., 2002) through heterochromatic silencing thought to occur through RNA-directed transcriptional silencing involving histone H3 methylation and heterochromatin formation (Stevenson and Jarvis, 2003). The mechanism of this type of RNA interference has been suggested by Volpe et al. (2002). Piwi-interacting RNAs, which utilize piwi-interacting RNA complex (piRC) have been isolated in rat testes (Lau et al., 2006) and *Drosophila* (Saito et al., 2006) and are implicated in posttranscriptional gene silencing (Carthew, 2006) and defense of genomes from transposons (Saito et al., 2006; Aravin et al., 2007).

RNAi in trypanosome genome studies

Characterization of putative coding regions is a prerequisite for converting raw genomic sequence data into biologically relevant information (Gopal et al., 2003). With the completion of trypanosome and leishmania genome projects (Berriman et al., 2005; El-Sayed et al., 2005a; Ivins et al., 2005), a wealth of information is available at the genome database at GeneDB (<http://www.geneDB.org>) (Arnaud Kerhornou and The Sanger Institute Pathogen Sequencing Unit, 2002; Hertz-Fowler et al., 2004). This can be exploited using techniques such as RNAi, which has been shown to occur in *Trypanosoma brucei* (Ngô et al., 1998) and *T. congolense* (Inoue et al., 2002) but not in *T. cruzi* (DaRocha et al., 2004) or *Leishmania spp.* (Robinson and Beverley, 2003).

RNAi in *T. brucei* was first reported in abstracts presented by Elisabetta Ullu and co workers in the Molecular Parasitology Meeting at Woods Hole, MA, in 1997. Ullu et al., (2004) have given a comprehensive review of RNAi mechanism in *T. brucei*. The RNAi technique is used to knock down the expression of a specific mRNA in African trypanosomes for the purpose of examining the function of its genes (DaRocha et al., 2004). It circumvents the

necessity of generating double knockouts, which is a requirement for such studies in diploid organisms; hence it is a simpler and faster method. Additionally, there is no need of prior knowledge on the chromosomal location of gene(s) under study. It works well in *T. brucei* due to the presence of Dicer (Shi et al., 2006) and a member of argonaute (AGO) protein family, TbAGO1 (Durand-Dubief and Bastin, 2003; Shi et al., 2004). The RNAi mechanism is also functional in *T. congolense* (Inoue et al., 2002). Since this finding, more has been done on utilization of this technique in trypanosome gene functions studies (Wang et al., 2000; Morris et al., 2001; Hendriks et al., 2003), development of cell lines (Wirtz et al., 1999; Chen et al., 2003; DaRocha et al., 2004; Alibu et al., 2005; Alford et al., 2005), construction of plasmid vectors for RNAi delivery (Wirtz et al., 1999; Wang et al., 2000; LaCount et al., 2000; Gull et al., 2002; Wickstead et al., 2002; Motyka et al., 2004; DaRocha et al., 2004; Alibu et al., 2005), *in vivo* analysis (Tschudi et al., 2003), RNAi library construction (Morris et al., 2002; Drew et al., 2003; Englund et al., 2005) and designing siRNAs (Redmond et al., 2003). Redmond et al., (2003) have developed a web-based tool for designing RNAi targets in *T. brucei*. This *T. brucei* functional genomics project (TrypanoFAN: <http://trypanofan.path.cam.ac.uk/trypanofan/main/>) aims to utilise the information from the *T. brucei* genome project to produce a research resource and systematic collection of mutants by targeted gene inactivation using RNAi. Trials by Subramaniam and coworkers (2004) on open reading frames (ORFs) in chromosome 1 have demonstrated its applicability as a robust and systematic functional annotation tool. These developments are important in the utilization of genome sequence for valuable functional studies and drug target discovery and validation.

Vehicles of siRNA delivery in trypanosomes

The success of RNAi in potential drug target discovery and validation depends on the efficient delivery of siRNA *in vivo*. Principally, two modes of delivery (plasmid/vector-based and chemically synthesized siRNA) are utilized. Direct siRNA transfections have the advantage of high efficiency and immediate initiation of gene silencing when compared to plasmid DNA transfections. Plasmid based siRNA on the other hand allows storage of trypanosomes with inducible RNAi and is much less expensive.

In trypanosome research, two types of plasmid-based double stranded RNA expression constructs have been developed. The first type has a single inducible promoter driving head to head arranged gene fragments (Ngô et al., 1998; Shi et al., 2000) (Figure 1, panel A), while the second has two opposing inducible promoters controlling the expression of a single copy gene fragment (Wang et al., 2000; LaCount et al., 2000) (Figure 1, panel B). Additionally, they have another promoter that drives the

expression of a selectable marker (Biebinger et al., 1996; Wirtz et al., 1999), usually a gene that confers antibiotic resistance. Where two opposing bacteriophage T7 promoters are used, control of expression is by binding of the tetracycline (*tet*) repressor to *tet* operators (Wang et al., 2000; Morris et al., 2001; LaCount et al., 2002). Examples of this type of inducible RNAi vectors are pZJM (Wang et al., 2000) and p2T7^{Ti} (LaCount et al., 2002). They are widely used because they allow a single ligation of PCR-generated fragments of any target gene between the opposing tetracycline-inducible T7 promoters (Wang et al., 2000; Motyka et al., 2004). Transcription of the insert in both directions is activated by induction with tetracycline.

Type two constructs are the stem-loop vectors and include pLEW79, pLEW100, pHD1145 (for procyclics), pHD1146 (for blood stream forms) and pHD1336 (Wirtz et al., 1999; Wang et al., 2000; Estévez et al., 2003). They involve cloning of the insert twice in opposite direction on either sides of a 'stuffer' fragment. The transcription of the (target)-stuffer-(reverse-target) construct generates a stem-loop RNA that mediates RNA interference (Alibu et al., 2005). A linearized construct allows integration by homologous recombination into the ribosomal DNA (rDNA) spacer region, a transcriptionally inactive segment of the *T. brucei* genome (Morris et al., 2001; LaCount et al., 2002; Motyka et al., 2004; Alibu et al., 2005). Transcription occurs on induction with tetracycline, hence producing mRNA homologs to the target the gene.

Challenges of RNAi in trypanosome research

Various limitations have been experienced in RNAi in trypanosomes. These generally involve the generation and maintenance of recombinants. First, "background" expression from the "repressed" tetracycline-inducible opposing T7 promoters affects selection of suitable transformants (Wirtz et al., 1998; Wang et al., 2000; Chen et al., 2003; Alibu et al., 2005). This has been an important limitation to knockdown studies of several genes essential to trypanosomes (Chen et al., 2003; Alibu et al., 2005). To overcome this, the use of stem loop constructs under the control of tightly regulated inducible EP promoters has been undertaken (Shi et al., 2000; Alibu et al., 2005). This approach however requires several cloning steps and some sequence combinations resist cloning (Drozd et al., 2002; Alibu et al., 2005). In addition, the EP promoter is down regulated in blood stream forms (Biebinger et al., 1996; Alibu et al., 2005). To mitigate against this, Alford and colleagues (2005) have developed bloodstream cell lines that exploit the strengths of inducible rRNA promoter.

The second limitation involves the development of RNAi revertants through deletion of target gene inserts (Chen et al., 2003). No appropriate solution has been achieved to this problem. However, precautionary mea-

asures taken involve the use of stem loop vectors, clonal RNAi populations (Motyka et al., 2004) and avoiding selection of transformants over long periods of time since the revertants begin to occur after the third day of induction (Alford et al., 2005).

The third limitation is the variation in regulation of expression of constructs due to availability of various sites for integration (Biebinger et al., 1996; Alford et al., 2005) and integration into an unintended region (Motyka et al., 2004). In the latter, Motyka et al. (2004) observed a change in orientation of dual T7 promoters in non stem-loop vectors resulting in transcription of distant genes. Since regulation is chromosomal and position dependent (Biebinger et al., 1996), variations occur in the clones generated. To circumvent this position effect, Alford et al. (2005) propose using a locus tagged with a unique sequence that allows constructs to be reproducibly inserted into the same genomic location. In their attempts to use this approach, they reported an advantage in reducing the amount of DNA used for transfection from 10µg to ~5µg. In addition, they developed a new blood stream cell line that allows high efficiency DNA-mediated transformation, addressing the fourth limitation of difficulties in generating recombinant bloodstream-forms.

The fifth limitation is the possible integration of the constructs in unanticipated regions rather than the transcriptionally inactive rDNA locus hence transcriptional up-regulation of the genes upstream and downstream of the integration sites as observed by Motyka et al., (2005). This knockdown affects more than one protein. They suggested a complete *Not I* digestion of constructs prior to transfection to avoid integration via circular plasmid and confirmation of integration sites in the clones developed.

The sixth shortfall is the inability to achieve a complete abolishment of gene function (Balana-Fouce and Reguera, 2007) hence the undetectable levels of the respective protein present (Motyka et al., 2004) can rescue the cells from potential lethal effects. Therefore, resulting phenotypes with no significant changes in morphology or organelles organization or growth rate, do not necessarily depict an unimportant gene in viability. In addition, high expression levels and stability of the some protein enables residual effects, resulting in false negative phenotypes. To overcome this shortfall, the development of live-cell reporter strains suggested by Subramaniam *et al.* (2004) could be an option for improving sensitivity and better interrogations. Precautionarily, RNAi experiments on both procyclic and bloodstream forms of the parasites should be encouraged since certain genes are developmentally regulated.

These drawbacks indicate that the success of various studies is pegged back by important limitations. Hence, efforts to develop an optimized RNAi toolkit that will allow an exhaustive analysis of gene function in trypanosomes need to continue. This would significantly enhance our knowledge of the biology of other members of disease

causing kinetoplastids, *T. cruzi* and *Leishmania spp.* which share 76% of genes with *T. brucei* (El-Sayed et al., 2005b).

The RNAi Library

Englund et al. (2005) have developed an RNAi library as a forward genetics approach to high throughput screening of trypanosomes genes. This involves the cloning of short random genomic fragments (approximately 0.7 kb) into a double T7 vector, transfection, generation and selection of clones with desired phenotypes. They applied this approach successfully in discovery of new genes for kDNA replication. It opens a wide window for a speedy characterization of genes responsible for important phenotypes and those essential for viability, leading to the identification of novel potential drug targets and biochemical processes. This overcomes the approach of reciprocal BLAST searches used in identifying orthologous and paralogous genes that are limited when the nature of proteins or pathways are unknown, as is the case for many biological processes in trypanosomes.

The RNAi library approach has a number of limitations, especially with regard to choosing the appropriate inserts. First, in cases where fragments have a *Not I* restriction site(s), integration of the constructs could occur in undesired sites as explained above. Second, it will be difficult to generate appropriate insert sizes (about 500bp) from digested total genomic DNA hence high possibilities of non-specific knockdowns. Third, though ideally rare, the criteria of not including the initiator ATG codon but using the region close to it hence the risk of forming a truncated protein is a possibility (Clayton et al., 2005). Fourth, generation of a library that covers a 26 megabase genome (Berriman et al., 2005) is not easily achievable because large numbers of recombinant trypanosomes will be required to cover a small percentage of the genome.

RNAi library approach should thus be applied with great caution but its use and improvement will be invaluable in genomics studies. One potential application could be to develop a cDNA based RNAi library similar to yeast two-hybrid cDNA library. This would allow investigation of genes expressed during specific developmental stages, and offer an alternative to the use of microarrays, which require considerable skill and the investment.

RNAi and drug target validation in trypanosomiasis

African trypanosomiasis remains among the most neglected tropical diseases in terms of drug development (Morel, 2003; Fairlamb, 2003; Pink et al., 2005). With none of the current control strategies being completely effective and resurgence of the disease (Legros et al., 2002), there is a great need for improvement of current approaches and development of new ones. Chemo-

therapy, which remains the mainstay for control is becoming increasingly compromised by drug resistance and toxicity (Verlinde et al., 2001). This is due to continued use of the few available drugs, which have resulted into multiple resistance (Matovu et al., 2001; Geerts et al., 2001), yet development of new drugs remains extremely slow.

Developing a new drug involves the identification and early (pre-clinical) validation of novel biological targets, a process termed target discovery (Lindsay, 2003; Knowles et al., 2003). In trypanosomes, various potential drug targets have been investigated. They include glycolysis (Verlinde et al., 2001), redox metabolism (Hunter et al., 1992; Stoll et al., 1997; D`Silva et al., 2000), purine metabolism (Chowdhury et al., 1999; Soulère et al., 1999), glycosylphosphatidylinositol (GPI) synthesis and myristate (Ferguson et al., 1999; Kimberly et al., 2001; Smith et al., 2004), fatty acid and sterol metabolism (Urbina, 1997; Urbina et al., 2002; Roberts et al., 2003), RNA editing, gene expression (Denker et al., 2002) and RNA splicing machinery (Denker et al., 2002; Owino et al., 2006). Barrett et al., (1999) and Wirth (2001) have given comprehensive reviews on potential drug targets in trypanosomes and leishmania. Using RNAi, a better understanding of these processes can be achieved. In addition, our knowledge of some of the processes has greatly improved. For example, based on the robustness of the technique, trypanosomes have been shown to synthesize fatty acids using the enzyme elongase (Lee et al., 2006) rather than a type II synthase (Kimberly et al., 2001), showing the potential to identify significant differences between parasite and host.

Comparative analysis of the trypanosome genome and those of hosts (or their relatives) can allow discovery of new targets, including genes or processes that are either absent in hosts (trypanosome specific) or, if present, are either sufficiently different to allow selective inhibition or non-essential for host survival. Switching off these genes by RNAi is an approach that can be used to determine if they are essential for growth or survival of trypanosomes, hence informing on their potential as targets for control.

Conclusion

The availability of the trypanosome genome sequence allows better understanding of known drug targets and identification of new targets. With clues of gene function available through annotation, a lot more should be done towards the conclusive determination of biological functions of these genes with priority to those that could be potential drug targets. This can be achieved using RNA-mediated RNA interference, which is fast and does not need prior knowledge of gene function. RNAi offers new hope for high throughput screening of the trypanosomes genome, with the potential discovery of new targets that can result in tools for control.

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