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RNA interference in protozoan parasites and its application

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ABSTRACT

RNA interference (**RNAi**) is a biological process in which RNA molecules are involved in sequence-specific suppression of gene expression, via small RNA triggers derived from double-stranded RNA that can target specific genes; it is a natural process that plays a role in both the regulation of protein synthesis and in immunity. Discovery of RNAi by Fire and Mello in 1998 had a profound impact on unraveling novel aspects of eukaryotic biology. RNA interference (RNAi) has proven to be an immensely useful tool for studying gene function and validation of potential drug targets in almost all organisms. A great advance in parasitic protozoa was achieved by the experimental demonstration of RNAi in *Trypanosoma brucei*, and in other protists such as *Leishmania braziliensis, Entamoeba histolytica* and *Giardia lamblia/intestinalis*. These organisms exhibit numerous differences beyond the core 'dicer' and 'slicer' activities, thereby expanding knowledge of the evolutionary diversification of this pathway in eukaryotes. When present, RNAi has led to new technologies for engineering powerful and facile knockdowns in gene expression, revolutionizing biomedical research and opening clinical potentialities. In this review, we discuss the distribution of RNAi pathways, their biological roles, and experimental applications in protozoan parasites.

1. Introduction

Parasitic protozoans are a highly diverse, evolutionary unrelated group that are taxonomically divided amongst at least five super groups or eukaryotic kingdoms.[1] Protozoan parasites are adapted to invade and live within the cells and tissues of other host organisms.[2] The dissemination of protozoan parasites through host tissues is hindered by cellular barriers, dense extracellular matrices and fluid forces in the bloodstream.[3] Parasitic protozoan infections represent a major health burden in the developing world and contribute significantly to morbidity and mortality. These infections are often associated with considerable variability in clinical presentation. Common protozoan diseases include malaria,[4] amoebiasis,[5] giardiasis,[6] toxoplasmosis,[7] trichomoniasis,[8] leishmaniasis,[9] and South American[10] and African trypanosomiasis,[11] of which malaria, trypanosomiasis, and leishmaniasis are considered the most important diseases that with the highest rates of morbidity and mortality. Chemotherapeutic drugs are the most effective medications used to treat these diseases but cause a plethora of side effects. Due to the lack of effective vaccines and the paucity of reliable drugs, there is an urgent need to develop new regimens for reducing the severe health risks associated with infection and the treatment of neglected tropical diseases.[12,13] (see Table 1)

RNAi refers to the mechanism in which double-stranded RNA (dsRNA) molecules cause sequence-specific mRNA degradation of a target gene and prevent mRNA translation, resulting in endogenous or exogenous target gene silencing typically termed a 'knockdown' of gene expression.[14] RNAi is initiated by various dsRNA 'triggers', which are enzymatically cleaved to generate small duplexes that program the AGO1 (slicer) complex for gene silencing (Fig. 1). First, a long RNA duplex (dsRNA) molecule is processed by Dicer (a specialized ribonuclease (RNase) III-like enzyme in the cytoplasm), producing small dsRNA-small interfering RNA (siRNA) duplexes of about 19–28 nucleotides, depending on the species. The siRNAs then interact with and activate the RNA-induced silencing complex (RISC). The endonuclease Argonaute (AGO) component of the RISC cleaves the passenger strand (sense strand) of the siRNA while the guide strand (antisense strand) remains associated with the RISC. Subsequently, the guide strand guides the active RISC to its target mRNA for cleavage by AGO. As the guide strand only binds to mRNA that is fully complementary to it, siRNA causes specific gene silencing.[15] Beyond the core canonical RNAi

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activities encoded by Dicer and AGO, a number of proteins are typically required for efficient RNAi, the nature and number of which depends on the species.

RNAi has been harnessed in the field of biotechnology for gene silencing and gene knockdown. The technology has had a profound impact on unraveling novel aspects of eukaryotic biology, agriculture, and medicine. Here we review RNAi pathways and RNAi biology in protozoan parasites and discuss the current and potential usefulness of RNAi as a tool for gene function studies and drug target analysis. We focus on the protozoan parasites whose RNAi pathways have been investigated thoroughly and are highly relevant to human disease. Some parasites such as *Trypanosoma brucei, Leishmania braziliensis, Entamoeba histolytica, Giardia lamblia* have canonical RNAi pathways and some organisms such as *Trichomonas vaginalis, Toxoplasma gondii* have at best only non-classical or even a complete lack of RNAi machinery. Recent studies have computationally identified putative micro RNAs (miRNAs) in protozoan parasites, including *G. lamblia*,[16] *T. gondii*,[17] and *T. vaginalis*[18] however, the functional relevance of these miRNAs has not been established and as of yet no miRNA-specific machinery has been identified within these genomes. It should be emphasized that an organism producing small RNAs or predicted miRNAs does not fulfill the functional criteria for having an active RNAi pathway. Here we consider functional siRNAs to be those that associated with AGO/slicing machinery and that program RNA degradation activity. We also discuss the evolutionary loss of the RNAi pathway in protozoan parasites such that RNA viruses may play a selective/driving force for the loss of RNAi.

2. Discovery RNAi pathways in protozoan parasites

2.1. Trypanosoma brucei

The first discovery of RNAi activity in protozoan parasites was in *T. brucei*,[19] contemporaneously with the first reports of RNAi in plants and worms in 1998 by *Andrew Fire and Craig Mello*. Introduction of exogenous or transgenic dsRNAs led to very specific suppression of target gene activity, opening the door for functional genomics. Mechanistically, a genetic knockout of *Tb*AGO1 leads to a loss of RNAi activity against target genes, and other phenotypes include loss of siRNAs derived from exogenous or transgenic double-stranded RNAs, and increased endogenous retroposition, establishing that the AGO1 function is required for all aspects of RNAi in trypanosomes.[20] Through genetic and biochemical approaches, Ullu and Tschudi's group elegantly demonstrated that there are two arms of the RNAi pathway in trypanosomes: one located in the nucleus and initiated by nuclear *Tb*DCL2[21] and the other located in the cytoplasm and initiated by cytoplasmic *Tb*DCL1.[22] Both arms feed into the single AGO1 protein.[20,23] The nuclear pathway is focused on the downregulation of transcripts derived from retroposons and repeated sequences, thereby helping to maintain genome stability.[24] In addition to the previous characterization of AGO1, DCL1 and DCL2, respectively, Barnes et al. identified RNA interference Factors 4 and 5 (*TbRIF*4 and *TbRIF*5) as a minimal set of core RNAi machinery in *T. brucei*.[25] It is likely that other cellular proteins participating in the RNAi pathway remain to be discovered.

There are various methods for developing the RNAi vectors used to achieve the desired downregulation of gene expression. Initially, a silencing construct encoding hairpin RNA (hpRNA) using a stem-loop vector was developed in *T. brucei*.[32] An alternative system is the inducible dual T7 promoter vector (pZJM) that allows for rapid cloning of genes into the vector.[33]

2.2. Leishmania (Viannai) braziliensis

Despite the success with *T. brucei*, unexpectedly studies of RNAi in the related trypanosomatids *L. major* and *T. cruzi* failed to show experimental RNAi activity, and several well controlled negative reports were published.[26,27] As parasite genomes rapidly emerged in the early 2000s, and the genes associated with RNAi were identified, inspection of the *T. cruzi* and 'higher' *Leishmania* (subgenus *Leishmania*) species revealed the absence of critical RNAi genes such as DCLs and AGO,[28] confirming the negative experimental results. Unexpectedly at the time, the genome of *L. braziliensis* (subgenus *Viannia*) contained clear orthologs of *T. brucei* AGO1, DCL1 and DCL2[29] suggesting this *Leishmania* subgenus might retain a functional RNAi pathway. This was confirmed experimentally by Lye et al. in 2010 for *L. braziliensis* and *L. guyanensis*.

RNA sequencing of *L. braziliensis* revealed the presence of small RNAs of the expected sizes and immunoprecipitated along with AGO1, confirming their functional involvement as seen with authentic siRNAs in *T. brucei* and higher eukaryotes.[30] Interestingly the *T. brucei* genome codes for a homologue of HEN1, a methyltransferase modifies the terminal ribose of siRNAs,[35] thus protecting the 3′ end from nuclease attack. However, HEN1 is not present in *Leishmania braziliensis*,[30] and the *L. braziliensis* siRNAs show significant 3' heterogeneity unlike *T. brucei*.[31] This suggests a mechanistic diversification of the RNAi pathway among the trypanosomatid protozoa, the significance or consequences of which are not yet understood.

Inducible systems are not well developed in *Leishmania*, and exogenous dsRNA is much less efficient than in African trypanosomes,[36] so most approaches involve stable introduction of RNAi-active constructs.[35,36] This of course limits their use to non-essential genes, although failure to obtain transfectants can be preliminary presumptive evidence of gene essentiality. Yates et al. developed a more streamlined method that allows for the assembly of a complete targeting vector from all of its constituent parts in a single-step multi-fragment ligation.[34] Lye et al. developed vectors facilitating generation of long-hairpin or "stem-loop" (StL) RNAi knockdown constructs, using Gateway ™

Table 1
RNAi genes and classes of small RNAs identified in protozoan parasites There are classical and non-classical RNAi pathways in protozoan parasites.

Classical RNAi pathways					
Parasite	siRNAs	miRNAs	Argonaute	Dicer-like	Feature
Trypanosoma brucei	+	-	+	+ **(two)**	AGO1, DCL1, DCL2, RIF4 and RIF5 as minimal set of core RNAi machinery[25]; A homologue of HEN1- methyltransferase modifies the terminal ribose of siRNAs[31]
Leishmania braziliensis	+	**?**	+	+	Does not code for a HEN1 RNA 2'-Omethyltransferase, which modifies small RNA 3'ends[30]
Giardia lamblia	+	+	+	+	Presence of snoRNA, a novel precursor of miRNAs[49]

Non-classical RNAi pathways					
Parasite	siRNAs	miRNAs	Argonaute	Dicer-like	Feature
Entamoeba histolytica	+	**?**	+ **(three)**	**? (atypical)**	Lacks of the domain architecture of classical Dicer enzymes[43]
Trichomonas vaginalis	+	+	+ **(two)**	+	Putative mRNA was identified through genome-wide surveyed[18]
Toxoplasma gondii	+	+	+	+	*Tg*-AGO belongs to the Ago-like family suggesting that the protein diverges significantly from its metazoan and plant counterparts[17]

site-specific recombinase technology, thus producing a powerful tool for studying *Leishmania* gene function with some unique advantages.[35,36] Similar Gateway@ compatible vector for gene silencing were developed by **the** Phillips's group for use in *T. brucei*.[37] These newer vectors facilitate high-throughput applications for gene silencing and provide tools for functional genomics in *L. braziliensis*. The stem-loop (StL) RNAi construct approaches were tested for knockdown activity[36] in which several endogenous genes–including lipophosphoglycan (LPG2 and LPG3), hypoxanthine-guanine phosphoribosyl transferase (HGPRT), and paraflagellar rod (PFR1 and PFR2) were knocked down, thus showing reductions in RNA function and establishing the activity of the RNAi pathway broadly across *L. braziliensis*.[36]

Many *Leishmania* (*Viannia)* parasites harbor the double-stranded RNA virus *Leishmania* RNA virus 1 (LRV1), which has been associated with increased disease severity in animal models.[38] Remarkably, LRV1 survives in the presence of the active normal endogendous RNAi pathway, which, in many organisms, can act to control RNA viruses.[39] Brettmann et al. reported significant levels (0.4–2.5 %) of small RNAs derived from LRV1 in both *L. braziliensis* and *L. guyanensis*,[40] establishing that LRVs are targeted to some extent in normal development. The most likely explanation is that virus retention must be maintained by a balance between RNAi activity and LRV1 replication. Interestingly, the typical StL vectors used in *Leishmania* lead to massively high levels of siRNAs, which when targeted against LRVs can lead to their elimination. This provides a useful tool for generation of virus-deficient lines for subsequent study, typically after removal of the StL construct.[40]

2.3. *Entamoeba histolytica*

The first evidence that expression of dsRNA can trigger down-regulation of a target mRNA in this species was published in 2004.[41] An AGO protein and several potential Dicer homologs with a single RNase III domain were identified through BLAST searches, thus strengthening the supposition that a functional RNA pathway does exist in the *E. histolytica*.[42] Pompey et al. reported an *E. histolytica* RNase III which lacks the typical domain architecture of canonical or minimal Dicer enzymes while maintaining dsRNA processing activity, yielding small RNAs that mediate gene silencing via RNAi.[43] These data advance the understanding of small RNA biogenesis in *Entamoeba* as well as broaden the spectrum of non-classical Dicer enzymes that contribute to the RNAi pathway. Moreover, *E. histolytica* **was** reported to have multiple abundant small RNA populations, including an abundant 27-nt small RNA population, which has an uncommon 5′-polyphosphate structure.[44] This unusual feature indicates that the small RNA population is likely generated in a Dicer independent manner, as canonical Dicer activity should yield 5' monophosphates. The pathway for generation of 5′-polyP small RNA is currently unknown. Interestingly, since the 5′-polyP small RNAs are Dicer-independent, it is possible that the parasite could have evolved some currently unknown mechanism to manipulate gene expression without a Dicer enzyme. An unique feature of silencing in *E. histolytica* is that H3K27Me2 functions as an amebic repressive epigenetic mark associated with silenced loci which mediated transcriptional gene silencing.[45] Zhang and Singh reported that three *Eh*Ago proteins have distinct subcellular localizations and bind 27-nt sRNAs[46] and the most highly expressed Argonaute protein is *Eh*Ago2-2.[47] The three *Eh*Ago proteins show specific sRNA binding and expression of their localization change in response to stress.[46] To understand some of the unique aspects seen in *E. histolytica*, Zhang and Singh identified 43 protein components of *Eh*Ago2-2 RNA-induced silencing complex (RISC) with a broad range of functional activities.[47] Two proteins, NAP12 and NAP07, with nucleosome assembly protein (NAP) domains not previously observed in other RNAi systems were identified as novel core members of amebic RISC.[47]

2.4. *Giardia lamblia*

Two genes, Argonaute and a Dicer-like enzyme which are central to the RNA pathway were identified through genome sequencing.[48] Saraiya and Wangs' report showed that a small nucleolar RNAs (snoRNA) derived miRNA mediated translational repression in *Giardia*.[49]

2.5. *Cryptosporidium parvum*

Genome sequencing revealed no conventional genes for RNAi machinery in the Apicomplexan parasite *Cryptosporidium* ***parvum***,[50] and thus far no evidence that endogenous pathways are able to mediate RNAi or silencing has emerged.

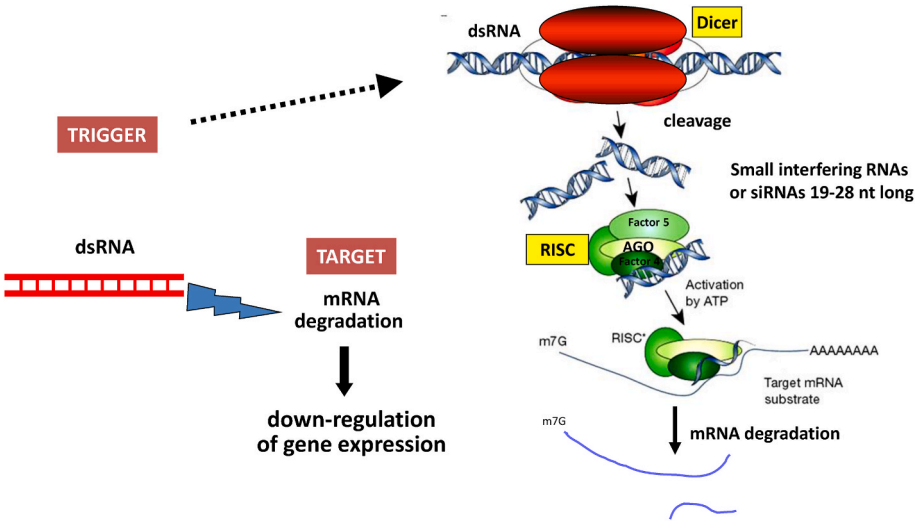

Fig. 1. Overview of the RNAi pathway RNAi works due to various dsRNA 'triggers' by generating small RNAs which then serve to program the AGO1 (slicer) complex for gene silencing. First, the Dicer-like enzyme processes a long RNA duplex (dsRNA) into small dsRNA-siRNA duplexes of about 19–28 nucleotides. The siRNA interacts with and activates the RNA-induced silencing complex (RISC). The endonuclease Argonaute (AGO) component of the RISC cleaves the passenger strand (sense strand) of the siRNA while the guide strand (antisense strand) remains associated with the RISC. Factors 4 and 5 were identified as minimal set of core RNAi machinery in *T. brucei*.[25] Subsequently, the guide strand guides the active RISC to its target mRNA for cleavage by AGO. Fig. was partially modified from.[52].

2.6. Plasmodium falciparum

There are no clearly identified RNAi genes were identified through database mining in apicomplexan parasites, *Plasmodium* ***falciparum***.[51]

2.7. Trypanosoma cruzi

Like ''higher' Leishmania species, *Trypanosoma cruzi genomes do not reveal the presence of homologs of key canonical RNAi pathway genes such as DCL and AGO, and well controlled functional tests show not evidence of RNAi activity*.[28,29,52]

3. The application of RNA interference to functional genomics in protozoan parasites

RNAi technology has shown **significance** from basic research to therapeutic applications. It has proven to be an immensely useful tool for studying gene function and validation of potential drug targets in almost **all organisms that possess functional pathway**. In this section, we particularly focus on the advance usefulness of RNAi technology in protozoan parasites.

3.1. Trypanosoma brucei

Following the initial discovery of RNAi in *T. brucei,* researchers developed RNAi technology as the method of choice for achieving downregulation of gene expression. The first genome-wide RNAi screen was performed in the laboratory of Paul Englund in a search for genes that modulate expression and posttranslational modification of surface coat proteins (EP procyclins) in *T. brucei*.[53] A systematic RNAi gene function screen for the entirety of chromosome 1 of *T. brucei* parasites was performed by analyzing 210 genes; RNAi knockdown of 33 % of the genes resulted in significant phenotypic differences, including defective growth and cell cycle progression and RNAi against 12 % of the studied ORFs was lethal.[54] A giant leap forward in the evolution of RNAi screening technology for trypanosomes was the development of the RIT-Seq (RNA Interference Target Sequencing) method in the laboratory of David Horn.[55] The strategy artfully combines the powers of genome-wide RNAi screens with the strength of Illumina genome sequencing for identifying all genes potentially associated with loss of fitness at the same time and within the same pool of cells. It relies on massive parallel sequencing of tags from the RNAi library present in the selected cell population to reveal gaps of genomic coverage that correspond to genes whose knockdown is detrimental to the parasite under the conditions tested.[55] Using this versatile new tool, Alsford and colleagues individually identified 1972 and 2724 genes with a loss-of-fitness knockdown phenotype in procyclic and bloodstream trypanosomes.[55] With improvements in efficiency of high-throughput phenotyping screens, RIT-seq represents a versatile new tool for genome-scale functional analyses and for the exploitation of genome sequence data. This revolutionary method has been used to link several hundred specific phenotypes with the downregulation of specific genes, including drug transporter genes, drug metabolism and activation mechanisms, quorum sensing genes, DNA replication and repair, and cell cycle progression.[56] Many investigations of *T. brucei* biology using RNAi screening have applied these advanced techniques. Important discoveries arising from RNAi screening include *PEX1*, essential for glycosome biogenesis and trypanosomatid parasite survival[57]; unique interactions of the small translocases of the mitochondrial inner membrane (TIMs) in *T. brucei*[58]; the role for a *T. brucei* cytosine RNA methyltransferase homologue in ribosomal RNA processing[59]; a conserved trypanosomatid differentiation regulator controls substrate attachment and morphological development in *T. congolense*,[60] and the identification of 30 transition fibre proteins in *T. brucei* reveals a complex and dynamic structure.[61]

3.2. Leishmania braziliensis

The stem-loop (StL) RNAi construct approaches were applied for knockdown activity in *L. braziliensis.* Several endogenous genes, lipophosphoglycan **synthesis gene 2 and 3** (LPG 2 and 3), hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and paraflagellar rod (PFR1 and PFR2) showed decreased levels of mRNA, confirming the utility of the RNAi pathway in *L. braziliensis*.[36] Amongst many examples, RNAi studies have confirmed the importance of polyphosphate (polyP polymerase vacuolar transporter chaperone 4) in the *Leishmania* life cycle.[62] Amastins are surface glycoproteins encoded by large gene families present in the genomes of *Leishmania* spp. Amastin knockdown in *Leishmania braziliensis* affects parasite-macrophage interaction and results in impaired viability of intracellular amastigotes.[63]

3.3. Entamoeba histolytica

By generating the plasmid library and inserting random fragments of gDNA into the pTriggerAdaptor plasmid, genes required for growth were identified[64]; hypothetical proteins play a role in stage conversion, activation of genes related to virulence and the stress response.[65]

3.4. Giardia lamblia

Maricial-Quino et al. developed a vector containing a cassette for the synthesis of double-stranded RNA (dsRNA), which can silence expression of a target gene through the RNA interference (RNAi) pathway.[66] The *G. lamblia* NADH Oxidase gene was silenced through this stem-loop RNAi approach.[66]

3.5. Toxoplasma gondii

Braun et al. 2010 reported that the highly divergent relatives of algal or fungal AGO1 and DCL were present in the *Toxoplasma* genome.[17] They speculated these might represent a patchwork gene network of RNAi silencing machinery, although noting that the phylogenetic support was weak. Sequencing of small RNAs mostly 25–27 nt showed broad mapping across non-coding intergenic regions, introns, protein coding regions, and a variety of DNA repeats and satellites. Computational screens suggested a handful of candidate miRNAs as well. A number of tantalizing parallel observations were reported, leading to speculations that *Toxoplasma* encoded a unique and highly divergent pathway with elements reminiscent of authentic RNAi or miRNA pathways of other organisms. However, thus far no experimental evidence has emerged confirming or establishing that these pathways are functional or of biological consequence.

4. Extension of RNAi technology to RNAi deficient protozoan parasites

Despite evidence that many parasites lack active, canonical RNAi pathway, the lure of this powerful technology has prompted a number of workarounds. Some involve direct application of small 'siRNAs', although further studies are needed to determine whether these act through an RNAi-like mechanisms, instead of the possibly more likely antisense RNA route which can also interfere with gene expression by specific or nonspecific mechanisms.[67–69] The desire for an experimental RNAi-like tool has prompted questions about whether the RNAi pathway could be restored in parasites in its entirety by provision of active genes from related species, as reported in budding yeast.[70] Thus far, these efforts have not been successful in protozoan parasites.

4.1. Trichomonas vaginalis

Due to lack of some parts of the required intrinsic RNAi machinery, the RNAi system is not functional in *Trichomonas vaginalis.* Ravaee et al.

used synthetic siRNAs to target two genes, α-actinin and cysteine protease 12 (*cp12*) to demonstrate how *T. vaginalis* cells might be amenable to RNAi approaches conducted by extrinsic siRNAs. It is unknown whether this may occur through canonical or noncanonical RNAi pathways, or something different.[67]

4.2. *Plasmodium falciparum*

Hentzschel induced gene knockdown in *Plasmodium by* introducing minimal, non-classical RNAi machinery components, solely requiring Argonaute 2 (Ago2) and a modified short hairpin RNA, AgoshRNA.[68] Through this strategy, the endogenous gene perforin-like protein was successfully silenced. The ability to render RNAi-negative organisms RNAi competent by just two components, Ago2 and AgoshRNA, is a useful paradigm that might find broad applicability in other species.

4.3. *Cryptosporidium parvum*

To circumvent the lack of endogenous pathways, Castelanos-Gonzalez developed a novel strategy to knock down *Cryptosporidium* genes by reconstituting the effector arm of the siRNA pathway.[69] By transfecting parasites with hybrid complexes formed between recombinant human Argonaute (hAgo2) and *Cryptosporidium* single-stranded RNA (ssRNA), several *Cryptosporidium* genes were proven to be knocked down.[69]

5. Conclusions and future perspectives

The studies above emphasize the utility of RNAi in a variety of protozoan parasites, and point to some workarounds for those species that lack demonstrable RNAi activity and/or canonical RNAi pathway genes. In this section we discuss the forces that might lead to the loss of RNAi in diverse protozoan lineages, and the relative merits and utility of RNAi approaches as new and even more powerful techniques deriving from the advent of CRISPR technology come into widespread use.

5.1. *Evolutionary consequences of the loss of the RNAi pathway*

Given the current view that the RNAi pathway originated deep in the ancestry of all eukaryotic cells, and its impact in diverse processes affecting gene expression and genome stability, it is perhaps unexpected that the canonical RNAi pathway has been lost multiple times in eukaryotic microbes, in fungi such as *Saccharomyces cerevisae* or *Ustilago*,[73] in apicomplexan parasites including *Plasmodium*, and several times in trypanosomatid protozoans including independently in *Trypanosoma cruzi* and *Leishmania* other than the subgenus *Viannia*.[74]

One argument rationalizing these events was that the adaptability and relative simplicity of microbial eukaryotes would allow them to more readily (at least over evolutionary time) tolerating the loss of RNAi. However, the known roles of RNAi and the consequences of its loss on gene expression and genome stability where studied suggests that loss of RNAi was unlikely to be completely neutral, but deleterious, to at least some extent. Presumably over time, compensatory alternative modes of gene regulation replacing RNAi-mediated pathways accumulated in RNAi-deficient lineages, Similarly, the impact on genome stability might be withstood under special circumstances, for example in the localization of known active transposable elements in *Leishmania* to telomeric regions or the large array of repeated genes encoding the splice leader in trypanosomatids.[29] Curiously, phylogenetic analysis shows that in *Leishmania* the loss of RNAi pathway could be mapped to the same lineage where loss of active transposable elements occur.[36] This raises an interesting chicken vs. egg challenge: did loss of RNAi open the way for loss of active transposable elements, or conversely, did loss of active transposable elements now permit loss of RNAi? Another possibility might be that somehow the processes of RNAi and transposition are in some fashion mechanistic linked, although currently there is no evidence supporting such a hypothesis.

5.2. *Selective forces favoring loss of RNAi in evolution*

Several hypotheses have been advanced to suggest that other some circumstances loss of RNAi may have selective value, potential compensating for other deleterious effects. It is not impossible that changes in gene expression pattern may in some circumstances prove beneficial – for example in the rise of *Viannia* sp. which exhibits numerous differences in biology, pathology and transmission relative to other *Leishmania* subgenera. Similarly, the genome instabilities associated with RNAi-deficiency potentially provide a new source for variation which may be of selective value under some circumstances.[28,29]

In *Leishmania,* it has been suggested that loss of RNAi in most species may account for the remarkable ability of these organisms to adapt by frequent generation of extrachromosomal circular elements.[75] Transcription around these typically occurs on both strands and generated antisense RNAs, unlike the normal chromosomal setting. Thus while RNAi proficient organisms will generate siRNAs targeting transcripts from extrachromosomal circular DNAs, RNAi-deficient organisms will fail to do this, thus allowing them to persist and function. Indeed, the identification of circular elements in RNAi-deficient *Leishmania* selected for drug resistance or other phenotypes is one rapid means for forward genetics.[72,76] In contrast, circular elements are rarely observed in RNAi proficient *Trypanosoma brucei*.[71]

It is well known that RNAi in many metazoan lineages plays key roles in viral defense, and a special case in *Leishmania* may involves the interactions between the RNAi pathway and persistent RNA viral elements, similar to a model proposed for the loss of RNAi in yeast species bearing dsRNA viruses.[70] Current data suggest that about 40 % of *Viannia* species isolated examined bear a dsRNA totivirus termed *Leishmania* RNA virus 1 (LRV1).[40] In studies in model animals the presence of LRV1 is strongly associated with hypervirulence, increased parasite numbers and metastasis, one hallmark of the more severe forms of leishmaniasis commonly seen in *Viannia* sp.[38] Thus, early in the evolution of *Viannia* sp. loss of RNAi could have selective value, in increasing both parasite numbers and/or transmission, and consequently, under some circumstances could favor loss of RNAi over retention.

It was recognized early on that RNAi has a widespread role in silencing parasitic nucleic acids, such as those of mobile elements and certain RNA viruses, and thus contributes to the maintenance of genome stability and to the prevention of viral spread. Viral invasion has been proposed as a selective force to drive loss of the RNAi pathway both in yeast species[70] and in Old World *Leishmania* species.[36,74] In one scenario, invasion by *Leishmania* RNA virus (LRV) at some point in *Leishmania* evolution could lead to an attenuation of the RNAi response, as many RNA viruses are prone to attack by cellular RNAi pathways. The challenge for this model is to explain what forces would prompt cells to favor RNA virus retention over disruptions arising from perturbation or loss of the RNAi pathway.[36]

5.3. *The utility of RNAi in the age of CRISPR*

As emphasized in this review, RNAi is a facile and powerful tool for studying the basic biology of cells, allowing the knockdown of gene expression to study protein function in a wide range of cell types. While under consideration in the expansion of RNA therapeutics, there are significant obstacles, such as off-target effects, potential toxicity and unsafe delivery methods.

More recently the emergences of genome editing tools such as CRISPR/Cas9 have had a major impact on the choices available to experimenters in probing gene function. One significant difference between RNAi and CRISPR/Cas9 is that RNAi reduces gene expression at the mRNA level (knockdown), while in the most common application CRISPR can completely and permanently silences the gene at the DNA

level (knockout). Many publications have compared to the advantages and disadvantages between this two technologies[77–80]

Nonetheless there are circumstances where RNAi may continue to offer some advantages. It is somewhat easier and more straightforward to target multi gene families by RNAi, especially if dispersed. Another and largely underexplored application of RNAi is in the rational engineering of 'hypomorphs', through introduction of dsRNA triggers of varying size but smaller than the threshold required for maximal activity. For example partial expression of an otherwise essential flagellar gene was used to generate a viable stable mutant of the *LbrIFT 140* gene in *L. braziliensis*, using a short 562 nt dsRNA trigger.[35] The study of hypomorphs may have advantages over off/on systems in some circumstances. Thus, the novel and sometimes unique properties of RNA interference in parasitic protozoans offers a fascinating system in which to explore both the evolutionary consequence of RNAi presence or loss, as well as offering new tools and approaches for dissecting the many pathways employed by parasites to grow, transmit and cause disease in their diverse hosts.[35]

CRediT authorship contribution statement

Lon-Fye Lye: Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Deborah E. Dobson:** Writing – review & editing, Methodology, Formal analysis. **Stephen M. Beverley:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **Min-Che Tung:** Writing – review & editing, Visualization, Validation, Formal analysis, Data curation.

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Ethics statement

Not required.

Data sharing statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Declaration of competing interest

The authors declare no conflict of interest.

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