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Synthetic siRNAs effectively target cystein protease 12 and α-actinin transcripts in *Trichomonas vaginalis*

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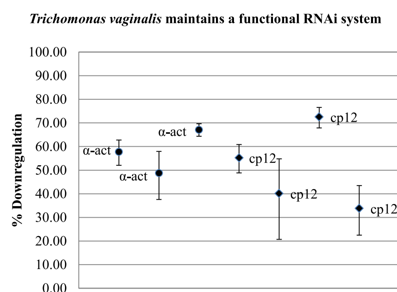
HIGHLIGHTS

- siRNAs tageting α-actinin and *cp12* in *Trichomonas vaginalis* were designed.
- Specific downregulation of α-actinin and *cp12* in *Trichomonas vaginalis* cells receiving siRNAs was observed.
- *Trichomonas vaginalis* maintains a functional RNAi system.

GRAPHICAL ABSTRACT



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ABSTRACT

The flagellated protozoan *Trichomonas vaginalis* (*T. vaginalis*) causes trichomoniasis, a reproductive tract infection, in humans. Trichomoniasis is the most common non-viral sexually transmitted disease worldwide. In addition to direct consequences such as infertility and abortion, there are indications that trichomoniasis favours development of prostate cancer and it has also been associated with increased risk of spreading human immunodeficiency virus and papillomavirus infections. Reports from around the world show that the rate of drug resistance in *T. vaginalis* is increasing, and therefore new therapeutic approaches have to be developed. Studying molecular biology of *T. vaginalis* will be quite helpful in identifying new drugable targets. RNAi is a powerful technique which allows biologist to specifically target gene products (i.e. mRNA) helping them in unravelling gene functions and biology of systems. However, due to lack of some parts of the required intrinsic RNAi machinery, the RNAi system is not functional in all orders of life. Here, by using synthetic siRNAs targeting two genes, i.e. α-actinin and cystein protease 12 (*cp12*), we demonstrate *T. vaginalis* cells are amenable to RNAi experiments conducted by extrinsic siRNAs. Electroporation of siRNAs targeting α-actinin or *cp12* into *T. vaginalis* cells resulted in, respectively, 48–67% and 33–72% downregulation of the cognate transcripts compared to the *T. vaginalis* cells received siRNAs targeting GL2 luciferase as a control. This finding is helpful in that it demonstrates the potential of using extrinsically induced RNAi in studies on molecular biology of *T. vaginalis* such as those aiming at identifying new drug targets.

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1. Introduction

Trichomoniasis, an infection caused by the protozoan *Trichomonas vaginalis* (*T. vaginalis*), is the second most common sexually transmitted infection in humans (Poole and McClelland, 2013). *T. vaginalis* is a flagellated, unicellular, anaerobic protozoan that infects individuals in the urinary tract, vagina and prostate (Kusdian and Gould, 2014; Schwebke and Burgess, 2004). Infection in women can result in urethritis, vaginitis, infertility, low birth weight and preterm delivery, but most infected men show no obvious symptoms. It has been suggested that trichomoniasis may increase the risk of Human Immunodeficiency Virus (HIV) and papillomavirus infections and transmission (Kissinger and Adamski, 2013), plus there are indications for association of this infection with increased risk of development of prostate cancer in men (Stark et al., 2009; Sutcliffe et al., 2006; Twu et al., 2014).

Nitroimidazoles such as metronidazole and tinidazole are the only drugs that are routinely used to treat trichomoniasis. However, drug-resistance is emerging so that up to 5% of cases in USA fail to respond to standard treatment protocols (Kirkcaldy et al., 2012). Similarly, over 17% of *T. vaginalis* isolated from cases in Papua New Guinea showed high level of resistance to metronidazole (Upcroft et al., 2009). Therefore, the need for developing new medication strategies for this disease exists.

Studying molecular biology of *T. vaginalis* can help identify new drug targets. Since the molecular biology of any organism is controlled by its functional genomics, it is obvious that identifying genes functions in *T. vaginalis* would prove helpful in this regard. By exploiting the RNA interference (RNAi) system (Fire et al., 1998), the expression of various genes can potentially be knocked down, and therefore their biological functions can be studied under controlled conditions. Establishment of a successful intracellular RNAi process is dependent on various cellular intrinsic factors such as the components of RNA Induced Silencing Complex (RISC) (Pratt and MacRae, 2009), and therefore only those cells that express all of the related factors are amenable to an RNAi experiment. In this line, while many protozoa have an active RNAi system (Kolev et al., 2011), it has been shown that some such as *Leishmania donovani*, *L. major* and *Trypanomsoma cruzi* lack a functional one (DaRocha et al., 2004; Lye et al., 2010; Robinson and Beverley, 2003). Here, we demonstrate that exposing *T. vaginalis* cells to extrinsic small interfering RNAs (siRNAs) can initiate specific gene down-regulation; a potential that can be exploited for RNAi - mediated experimental and therapeutic procedures.

2. Materials and methods

2.1. Cell culture

A common *T. vaginalis* isoform (named strain "F" for "Fars Province") isolated from cases in south of Iran and characterized by Dr. Gh. Hatam (co-author to this work) was used for this study. The cells were recovered from frozen samples and grown and maintained at 37 °C in TYI-S-33 medium containing 10% heat-inactivated adult bovine serum (cat# B9433, Sigma), 1% pen-strep (cat# P4333, Sigma) and 2% multivitamin mixture (cat# 318; Biofluids Inc. Rockwille). The medium was refreshed every 2–3 days and the culture was split before the cells reached the cell density of 1×10^6/ml. For the experimental procedures in this work, the cells from under ten subcultures were used.

Table 2
The sequences of the primers used in this work.

Gene name	Forward sequence (5′>3′)	Reverse sequence (5′>3′)
α-actinin	TTCTCGAAATCATCGGTAAG	TCACGAGCTGTAGCTTCTTC
cp12	TAGGATTTCAACTTGCTTCC	GTGCCTTGTACTCAGCTTGT
β-tubulin	TCCAAAGGTTTCCGATACAGT	GTTGTGCCGGACATAATCATG

2.2. siRNAs and primers

The sequences for α-actinin (TVAG_190450) and *cp12* (TVAG_410260) transcripts were retrieved from Trichomonas Genome Project data base http://trichdb.org/trichdb/ (Aurrecoechea et al., 2009) and siRNAs targeting these transcripts were designed using the Whitehead Institute siRNA design tool (Yuan et al., 2004). In order to maintain specificity and avoid off-target effects, the sequences of the selected siRNAs were evaluated using BLAST function at trichDB as well as the BLAST tool available at NCBI (www.ncbi.nlm.gov/BLASTn, USA). The designed siRNAs, and a control siRNA targeting firefly GL2 luciferase, were ordered from MWG, Germany. Table 1 shows the sequences of the siRNAs used in this project.

The primers used in PCR (including qPCR) reactions were designed using Primer3 tool available online (Rozen and Skaletsky, 2000). The selected primers were searched for any non-specific interactions using BLAST functions at NCBI and TrichDB. The primer sequences for the internal control (β-tubulin) were obtained from Simpson et al., 2007 (Simpson et al., 2007). The primers were synthesized by Sinaclone (Tehran, Iran). The sequences of the primers used in this work are presented in Table 2.

2.3. Electroporation

The day before electroporation a fresh culture was seeded at 3×10^5 cells/ml with growing *T. vaginalis* cells. The following day, when the cells were still at their exponential phase of growth (i.e. ~ 1×10^6 cells/ml), they were harvested by centrifugation at 2000 *g* for 30 min at room temperature. The cell pellets were re-suspended in fresh antibiotic-free complete TYI-S-33 growth media to a final cell concentration of 40×10^6/ml. For electroporating the cells, 270 μl of the cell suspension (containing a total of ~10×10^6 cells) was transferred to a 0.4 mm electroporation cuvette and then placed on ice for a few minutes. For each siRNA, 30 μl of 40 μM stock siRNA was added to a cuvette containing the cells (4 μM final siRNA concentration). The preparation was then gently mixed by flipping and quickly placed on ice. After ~10 min of incubation on ice, the cell preparation in each cuvette was gently mixed and immediately electroporated on a Bio-Rad gene pulser (Bio-Rad, USA) with the following setting: 320 v, 1000 μF, 750 α. The electroporated cells were immediately placed on ice and after ~10 min of incubation, they were added to 10 ml of antibiotic-free complete growth medium and transferred to a 37 °C incubator. Following ~3 h of incubation, Penicillin/Streptomycin was added to

Table 1
The sequences of the siRNAs used in this project.

Gene name	Sense sequence 5′>3′	Antisense sequence 5′>3′
α-actinin	GCCCAACAGAAAUCCGUAAdCdG	UUACGGAUUUCUGUUGGGCdTdG
cp12	GCGAAUUCCAGCUCUCAAUdTdT	AUUGAGAGCUGGAAUUCGCdTdT
GL2 Luciferase	CGUACGCGGAAUACUUCGA_dTdT	UCGAAGUAUUCCGCGUACGdTdT

the culture at a final concentration of 100 U/100 μg/ml, respectively, and the cells were incubated for a total of 24 h at 37 °C before being harvested for RNA extraction. Each experiment was performed in triplicate each time and the whole procedure was also independently repeated on separate occasions.

2.4. RNA extraction, DNase treatment and cDNA synthesis

For total RNA extraction the RNeasy®plus minikit (cat# 74134, Qiagen, Germany) that contained a built-in DNase treatment procedure was used. For each RNA extraction reaction, 4 × 10^6 cells were used. The RNA was extracted according to the instructions given by the manufacturer. The extracted RNAs were qualitatively, by running on agarose gel, and quantitatively, by spectrophotometry, evaluated and the RNAs of good quality were used for further experimentation. Before using for cDNA synthesis, RNAs were subjected to further DNase treatment using TaKaRa DNase I kit (Cat#, 2270A TaKaRa, Japan). 400 ng of DNase treated RNA was used to synthesize cDNA using Primescript™ RT reagent kit (Cat# RR037A, TaKaRa, Japan) according to the instruction provided by the manufacturer. The unused RNAs and cDNAs were stored at −80 °C and −20 °C, respectively.

2.5. qRT-PCR

SYBR®*Premix Ex Taq*TM II kit (cat# RR037A, TaKaRa, Japan) and a MiniOpticon™ Real-Time PCR System instrument (Bio-Rad, USA) were used for qPCR analysis. The cDNAs synthesized using 400 ng total RNA were diluted 1:5 in nuclease free dH2O and 1 μl from this dilution was used in each 15 μl qPCR reaction. The reactions were prepared in triplicates for each sample. qPCR was performed using the following protocol: 95/30sec, 40 × (95/5sec, 60/60sec); followed by a melting curve analysis. Initially, before running qPCR for gene expression analysis, a primer optimization analysis, for selecting the appropriate primer concentrations, and standard curve analysis, for determining the reactions efficiencies, was performed. Since the efficiencies were within the acceptable range (<10% of one another), ΔΔCt method was used to calculate the fold change in gene expression.

3. Results

3.1. *T. vaginalis* growth plot

Initially, the growth pattern of the *T. vaginalis* cells used in this work was determined. For this purpose, TYM complete media was seeded at 2 × 10^4 cells/ml with cells from an actively growing culture and incubated at 37 °C. The growth pattern of *T. vaginalis* was calculated by counting the alive *T. vaginalis* cells at various time points (Fig. 1). Based on this experiment the exponential growth phase of *T. vaginalis* starts at cell concentrations of ~5 × 10^4/ml (at 48 h after seeding) and cells reach the stationary phase at ~ 2 × 10^6 cells/ml.

3.2. α-actinin and cp12 knockdown by RNAi

T. vaginalis cells were transfected with siRNA oligonucleotides at their exponential growth phase as explained in M&M section. Three types of siRNAs i.e. siRNAs targeting α-actinin, siRNAs targeting *cp12* and control siRNAs targeting firefly GL2 luciferase were used in this experiment. 24 h after transfection, cells were checked for viability by light microscopy and trypan blue dye exclusion examination. This indicated minimal cell death and no obvious difference between the test and control groups were observed at this stage. At this time point total RNA was extracted (See **M&M** for details) and used for RT-qPCR. In independently replicated experiments, the *T. vaginalis* cells which had been transfected with either siRNAs targeting α-actinin or those targeting *cp12* showed, respectively, 48%–67% and 40%–55% downregulation in the respective mRNA expressions compared to the control groups that had received non-specific siRNAs targeting GL2 luciferase (Fig. 2). Since α-actinin is also considered as a house keeping gene, in a separate set of experiment we used this gene as a normalizer to evaluate *cp12* expression levels in cells that received siRNAs targeting *cp-12* or siRNAs targeting GL2 luciferase (as controls). This experiment also showed similar results by showing specific *cp-12* downregulation (33–72%) by siRNAs targeting *cp-12* (Fig. 2).

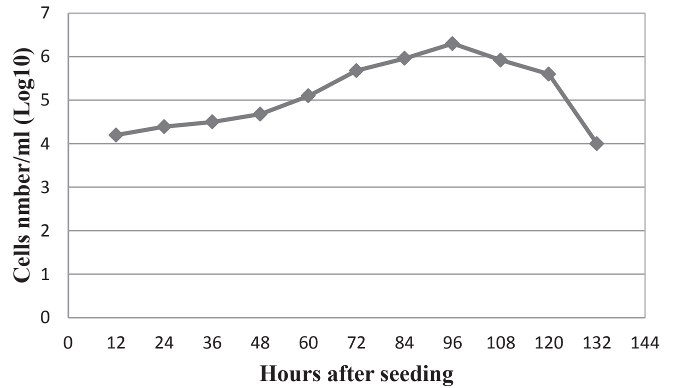

Fig. 1. *T. vaginalis* growth plot. *T. vaginalis* cells were counted at various time points (X-axis) after seeding (see M&M for details) to determine *T. vaginalis* growth rate (Y-axis). This experiment showed *T. vaginalis* cells remain at their exponential phase of growth until cell density of ~2 million cells/ml culture medium.

4. Discussion

Trichomoniasis is a common infection in humans standing at the second top most common sexually transmitted infection in the world (Johnston and Mabey, 2008; Poole and McClelland, 2013). Though this infection in men is mostly asymptomatic, but infected women may suffer from various degrees of illness ranging from vaginitis and metritis to infertility and abortion (Carne et al., 2013; Petrin et al., 1998; Sparks, 1991). The aforementioned maladies are

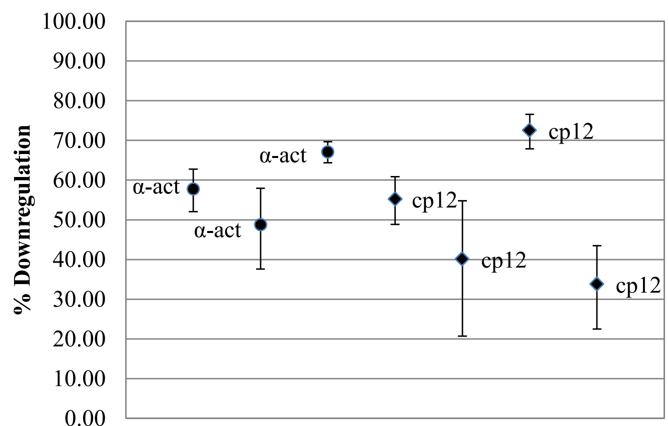

Fig. 2. Downregulation of α-actinin and *cp12* genes in *T. vaginalis* cells transfected with their specific siRNAs as determined by qPCR. This experiment showed that the expression level of α-actinin (α-act) or *Cp12* (cp12) was significantly (t-test *P* values 0.009 and 0.016, respectively) downregulated by their cognate siRNAs compared to cells received siRNAs targeting firefly luciferase as a control. Bars indicate downregulation levels ± 1 SD for each experiment of three replicates.

either directly caused by *T. vaginalis* itself or are resulted from the *T. vaginalis* - induced changes in the urogenital microenvironment in favour of persistence of other pathogens (Brotman et al., 2012; Fettweis et al., 2014; Hirt and Sherrard, 2015; Kusdian and Gould, 2014). Furthermore, it is believed that this infection may increase the transmission rate of HIV and papillomavirus (Kissinger and Adamski, 2013; Mirmonsef et al., 2012) and it has also been associated with increased risk of development of prostate cancer in men (Mitteregger et al., 2012; Stark et al., 2009; Sutcliffe et al., 2006; Twu et al., 2014). The routine and choice medication for treating *T. vaginalis* infection is by nitroimidazoles, but drug resistance is emerging (Kirkcaldy et al., 2012; Upcroft et al., 2009). Therefore, new preventive and/or therapeutic strategies are needed to be explored, and understanding the molecular biology of this organism should be very helpful in this regard. However, due to factors such as the limitations mentioned below, studies focused on molecular biology of *T. vaginalis* are, comparatively, scarce.

Reverse genetic approaches are routinely used to study the molecular functions of genes in various organisms. Homologous recombination strategy has been used to knock out genes in *T. vaginalis* (Bras et al., 2013; Land et al., 2004), but due to rather extensive genomic duplications in this organism (Carlton et al., 2007) and, hence, presence of expressed and functionally redundant paralogous genes (Gould et al., 2013) this approach does not always result in a phenotype (Land et al., 2004). Accordingly, in order to achieve the maximum expression knockdown ensued with a phenotype either all paralogous genes must be knocked out (mostly not feasible) or other strategies should be considered to target them all at once. Antisense technology has been exploited to target genes in *T. vaginalis* cells (Lama et al., 2009; Mundodi et al., 2007; Munoz et al., 2012; Ong et al., 2007), but the potentials and superior power of RNAi suggest it as a better approach for targeting the mRNAs expressed from different paralogous loci (Jover-Gil et al., 2014).

The rather newly discovered cellular RNAi system has proven to be a very powerful tool for functional genomics studies as well as for therapeutic-oriented approaches (Davidson and McCray, 2011; Ketting, 2011). Since 1998 when RNAi system was explained in *Caenorhabditis elegans* (Fire et al., 1998), the existence of such system in many other organisms, including parasitic protozoa, has been documented (Batista and Marques, 2011; Kolev et al., 2011). In a final stage of an RNAi process, a small RNA directs specific silencing of gene expression by various means such as degradation of the cognate mRNA, inhibition of translation or transcriptional silencing. In addition to this small interfering RNA, which endows the specificity to the process, RNAi is dependent on several intracellular factors such as RISC components without which no interference can happen (Kuhn and Joshua-Tor, 2013). Therefore, though it has been demonstrated that in many protozoa introducing double stranded RNAs into a cell is enough to trigger the RNAi machinery, but this may not be generalized (Kolev et al., 2011). Indeed, in some protozoa it has been demonstrated that due to lack of some of the RNAi system components, an effective silencing using RNAi cannot be established. For examples, it has been shown that *Leishmania braziliensis* has a competent RNAi system (Lye et al., 2010), but *L. major*, *L. donovani* and *Trypanosoma cruzi* lack some functional aspects of the RNAi system (DaRocha et al., 2004; Robinson and Beverley, 2003) and therefore this system cannot be used to experimentally target gene expression in these protozoa.

Functional genomics and bioinformatics studies suggest *T. vaginalis* has the genes for the proteins of argonaute family and the presence of miRNA genes and miRNA related activity has also been demonstrated in this organism (Huang et al., 2012; Lin et al., 2009a, 2009b). In order to experimentally verify whether synthetic siRNAs, as potential experimental and therapeutic tools, can direct specific gene silencing in *T. vaginalis*, we designed an RNAi experiment on *T. vaginalis* using synthetic siRNAs targeting α-actinin and *cp12*. After determining that these two genes are expressed in the *T. vaginalis* cells under experiment (data not shown), we targeted them for knockdown by transfecting *T. vaginalis* cells with the related siRNAs. We used a functional siRNA targeting firefly GL2 luciferase, but without a target in *T. vaginalis*, as a control. Our result demonstrated that α-actinin and *cp-12* were specifically and significantly (48%–67%, t-test *P* value 0.009, and 33%–72%, t-test *P* value 0.016, respectively) downregulated by their cognate siRNAs (Fig. 2). These findings demonstrate RNAi can be specifically triggered in *T. vaginalis* using extrinsic siRNAs and therefore, further experimental and therapeutic procedures in this protozoan using this system are possible.

It is worth mentioning here that the level of reduction in mRNA does not always, for various reasons such as long protein half life, correspond to a simultaneous and similar reduction in the protein level; plus, reduction in protein levels are, to some extent, tolerated by cells. Therefore, when an RNAi experiment for functional studies is conducted and observable changes in phenotype are expected, optimization of the RNAi experiments alongside with monitoring the protein levels are necessary. In this line, one can consider approaches that result in more stable knockdown such as taking advantage of short hairpin RNA (shRNA) expressing vectors.

Conflicts of interest

Author declare no conflict of interest.

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