CRISPR/Cas9 in Leishmania Genome Editing: Towards a Proof of Concept

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ABSTRACT

Leishmaniasis is a parasitic Neglected Tropical Disease (NTD) that affects mostly poor countries of the third world due to their limited access to clean water and health care. The disease is transmitted through the bite of a sand fly of the genus *Phlebotomus* in the Old World, and of the genus *Lutzomyia* in the New World. Historically, NTDs have not been given adequate attention or research funding and finding curative treatments has been slow. However, the fact that the insect vector and the parasite can live and thrive in any ecological system in the world, and under the current circumstances of the world’s worst refugee crisis playing a major role in the geographical distribution of the disease, leishmaniasis has been given more attention. Leishmaniasis is manifested in cutaneous or mucosal forms causing scarring, disfiguration, bleeding and breathing difficulties, or in a visceral form that causes liver and spleen malfunction, a compromised immune system and death if not treated. Currently, there is no curative treatment or vaccination for leishmaniasis. The available treatments aim to reduce morbidity from the cutaneous and mucosal leishmaniasis, and mortality from the visceral form. Finding new medicines for leishmaniasis requires identifying new drug targets, hence, gene function studies are essential for revealing potential specific drug targets in this parasite.

Although the whole genomes of various leishmania species have been sequenced, very little is known about the gene functions due to the absence of an RNAi pathway in the parasite and the difficulty of achieving homozygous knock out (KO) by the traditional homologous recombination methodology in a diploid organism like leishmania. The new CRISPR/Cas9 technology for targeted gene editing holds great promise for easier study of gene functions in leishmania, hence allowing identification of new drug targets.

Very few studies were done on applying CRISPR/Cas9 in Leishmania. Therefore, we attempted to adapt the CRISPR/Cas9 system to *Leishmania donovani* for a proof of concept. In this research we describe a new culture medium not typically used in the literature for *L. donovani* that allows optimal growth of promastigotes *in vitro*. Also we describe protocols for genomic DNA extraction as well as oligonucleotide
annealing and cloning conditions adjusted specifically for the generation of gRNA expression plasmids for leishmania. Furthermore, we established a stable Leishmania cell line expressing Cas9, and set up the basic strategy for applying the CRISPR/Cas9 gene knockout technology in *L. donovani*. After specifically targeting the ODC (ornithine decarboxylase) gene for KO, we found by DNA sequence analysis that no Insertion or Deletion (InDel) mutations occurred at the targeted site. This finding indicates that although the Cas9 enzyme is likely to scan and cut the doublestranded DNA frequently at the targeted site, the double strand breaks are being repaired accurately each time by an error free repair machinery such as Homologous Directed Repair. Thus, we propose as specific future direction the use of HDR for interruption of the gene open reading frame instead of relying on simple Non-Homologous End Joining for creating random InDels upon repairing the double stranded break by Cas9. Overall, this study paved the way for adapting the CRISPR/Cas9 system to genome editing in Leishmania, for future proof of concept and gene function studies, identification of new drug targets and possibly creating vaccinations by generating attenuated Leishmania strains.
MONTCLAIR STATE UNIVERSITY

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by

Zein Kasbo

A Master’s Thesis Submitted to the Faculty of
Montclair State University
In Partial Fulfillment of the Requirements
For the Degree of
Master of Science in Pharmaceutical Biochemistry

August 2017
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CHAPTER ONE

INTRODUCTION

1.1. Leishmaniasis: A Neglected Tropical Disease

Leishmaniasis along with other diseases such as lymphatic filariasis, dengue and trachoma are categorized as Neglected Tropical Diseases (NTDs). NTDs affect mostly poor countries of the third word due to limited access to clean water and health care. Hence, historically, NTDs have not been given adequate attention or research funding and finding curative treatments has been slow. These diseases take a tremendous toll on the life of patients, physically, psychologically and socially. Whereas the majority of NTDs are not lethal, Leishmaniasis can be. Leishmaniasis is manifested in cutaneous or mucosal forms causing scarring, disfiguration, bleeding and breathing difficulties, or in a visceral form that causes liver and spleen malfunction, a compromised immune system and death if not treated.

Finding new medicine for leishmaniasis requires identifying and evaluation new drug targets, hence, gene function studies are essential for revealing potential specific drug targets in this parasite. Although the whole genomes of various strains of leishmania have been sequenced very little is known about its gene function. This is due to the absence of an RNAi pathway in the parasite, thus allowing gene function studies only by the traditional and laborious targeted gene knock out (KO) by homologous recombination methodology (W. W. Zhang & Matlashewski, 2015). The advent of CRISPR/Cas9 for easier targeted genome editing that can potentially expand the possibility of studying gene function in leishmania. this would then allow translational research to identify new drug targets, and/or to create attenuated strains with multiple gene knock outs could form the basis for vaccination trials.
1.2. The Disease

Leishmaniasis is a vector transmitted NTD that can be caused by 20 different species of the protozoan parasite Leishmania. Depending on the infectious strain, leishmaniasis is manifested by a visceral form (kala-azar) that affects multiple internal organs such as the spleen and liver; a cutaneous form that results in skin sores, and a subcutaneous form that affects the mucous connective tissues. Of note is most people infected by the parasite do not develop any symptoms at all in their life, therefore, the term "leishmaniasis" refers to the fact of becoming sick due to a Leishmania infection and not the mere fact of being infected with the parasite. Cutaneous leishmaniasis (CL), a flesh-eating disease, is the most common form of leishmaniasis. CL is caused by the old world (Eastern hemisphere) leishmanial species Leishmania tropica, L. major, and L. aethiopica, as well as L. infantum and L. donovani, and the new world (western hemisphere) species including L. mexicana species: L. mexicana, L. amazonensis, and L. venezuelensis and the subgenus Viannia (L. braziliensis, L. guyanensis, L. panamensis, and L. peruviana). Infected patients develop skin sores that turn to volcano-like ulcers with raised edges and a central depression. Ulcers are generally painless but can be painful if infected with bacteria or if the ulcer is located near a joint. Skin sores are accompanied by swollen nearby lymphatic glands. Cutaneous lesions may self-heal or require treatment. The subcutaneous (mucosal) form of leishmaniasis (ML) may develop one to five years after a cutaneous infection due to mucosal dissemination of the parasite from skin lesions that were not treated, or that were treated suboptimally. Mouth, nose and lip lesions develop, and are accompanied by stuffy or runny nose, nose bleeding and breathing difficulty. If not treated, a subcutaneous infection can result in ulcerative destruction of the nasooropharyngeal mucosa. Visceral leishmaniasis (VL) is the most dangerous form of leishmaniasis. VL is caused by L. donovani and L. infantum. Clinical symptoms of VL include spleen and liver enlargement, high fever, weight loss, low red blood cell count (anemia), compromised immune system including low white blood cell and platelet counts. Patients exhibit abnormal blood test results including a high total protein level and a low albumin level, with hypergammaglobulinemia. Asymptomatic patients may exhibit clinical manifestations years after infection upon becoming immunocompromised.
due to other medical causes such as HIV co-infection. If untreated, VL is fatal either directly or indirectly due to complications such as secondary mycobacterial infection or hemorrhages.

The disease is transmitted from one infected person to another through the bite of an infected phlebotomine sand fly. The insect vector is tiny in size, only 2-3 mm long. Only female phlebotomine sand flies can transmit leishmaniasis, by feeding on human blood to obtain proteins needed for its egg development. There are around 500 phlebotomine species known worldwide, however, only around 30 species have been identified as being a significant vector of Leishmaniasis. Once uptaken by the insect vector, the parasite undergoes a major transformation that ranges between 4-25 days. After which, when the infected sand fly feeds again on a new source of blood, it inoculates the parasite into the new host through its proboscis, completing the transmission cycle (Figure 1.2).

1.3. Epidemiology

Leishmania parasites are found in about 90 countries in the tropical and subtropical areas as well as southern Europe. The parasite and its insect vector can live and thrive in a variety of ecological settings ranging from rain forests to deserts. Climate change and population migration play an important role in expanding the geographical area where leishmania and its insect vector are disseminated. According to the World Health Organization (WHO) report about the epidemiology situation of CL in 2015, Old world leishmania is most prevalent in Iran, Afghanistan, Syria and Algeria where the number of reported new cases in 2013 exceeded 5000. Bangladesh, Turkey, Iraq, Saudi Arabia, Yemen, Tunisia and Morocco have reported between 1000-4999 new cases in 2013. India, Bangladesh, Ethiopia, Sudan, South Sudan (Old world) and Brazil (New world) reported more than 1000 new cases in 2013. Somalia and Iraq reported between 500 and 999 new cases in 2013. In the new world (the Americas), CL is most prevalent in Brazil, Colombia and Peru where the reports indicate more than 5000 new cases in 2013. Panama, Nicaragua and Honduras reported between 1000-4999 new cases in 2013 (WHO, 2017).
Status of endemicity of cutaneous leishmaniasis, worldwide, 2013

Figure (1.1): Epidemiological situation of Leishmaniasis (WHO, 2013)
1.4. **Leishmania life cycle**

Leishmanial parasites cycle between two main life stages, promastigote and amastigote. The infective form of the parasite called promastigote is transmitted to the human host through the bite of infected female sand fly of the genus *Phlebotomus* in the Old World, or of the genus *Lutzomyia* in the New World (Figure 1.2: step 1). The parasite is phagocytized by macrophages and other mononuclear phagocytes and transforms into amastigotes. During the amastigote stage of life, the parasite is intracellular, non-motile, and its flagellum does not protrude further than the body surface and cannot be distinguished by light microscopy. The amastigote cell size is approximately 3-6 x 1.5-3.0 μm. *Leishmania* amastigotes live inside macrophages and multiply by longitudinal binary fission at 37°C (Figure 1.2: Steps 2-4). On the other side, a sand fly become infected with the parasite upon feesting on a blood meal from an infected individual (Figure 1.2: Step 5). In the gut of the insect vector, amastigotes develop into promastigotes at 27°C. The promastigote is an extracellular life stage, during which the parasitic cell is motile through an anterior body flagellum, and grows and multiplies by longitudinal binary fission. The promastigote cell size is roughly 15-30 x 5 μm. Once motile promastigotes migrate into the proboscis of the fly and become ready to infect a new vertebrate victim (Figure 1.2: steps 6-8).
Elimination of leishmanial infection from the body depends on a combination of factors including, but not limited to, the ability of leishmania to fight and evade the immune system, the immune status of the patient and possible co-infection with other pathogens such as HIV.

1.5. Diagnosis and treatment options

Diagnosis of visceral leishmaniasis is challenging because its clinical symptoms are similar to those of other diseases such as malaria and tuberculosis. VL is diagnosed by combining the observations of clinical manifestations with laboratory diagnosis, including parasite identification by light microscopy in stained specimens of relevance, parasite DNA detection in patient samples, and immunological testing. With VL, specimens are taken from the spleen or bone marrow to be examined, while in CL, they are taken from skin lesions. Under the light microscope, amastigotes appear in the cytoplasm of macrophages, where one can distinguish the nucleus and
the rod-shaped kinetoplast by Giemsa stain (CDC). PCR is used to detect the parasite DNA in patient samples. Primers targeting conserved sequences on the minicircles of Kinetoplast DNA, allow not only the detection of the parasite, but also species identification (Célia Maria Ferreira Gontijo; Melo Norma Maria, 2002).

There is no vaccine, nor a curative treatment available for leishmaniasis. Current therapy is attempted to lower mortality in visceral leishmaniasis, and morbidity in both cutaneous and mucosal leishmaniasis. The only Food and Drug Administration (FDA) approved drugs for leishmaniasis are liposomal amphotericin B (L-AmB) which is delivered intravenously for the treatment of visceral leishmaniasis and miltefosine which is delivered orally for the treatment of cutaneous, mucosal and visceral leishmaniasis, though both these treatments have a high risk of side effects such as reproductive toxicity, ototoxicity, liver and renal failure. However, these treatments are expensive and often unaffordable in poor countries. This is further compounded as cheaper generic drugs are less effective and require lengthy treatments with more toxic side effects. For prevention of leishmaniasis in travelers, no vaccines nor chemoprophylaxis are currently available; personal protective measures to minimize exposure to sand fly bites are recommended. Taken together, there is an urgent need for new affordable anti-leishmanial medicines and chemoprophylactic agents for the eradication and prevention of this severe disease (Aronson et al., 2016).

Finding new medicine for leishmaniasis requires identifying and evaluating new drug targets, hence, gene function studies are essential for revealing potential and specific drug targets in this parasite. Although the whole genomes of various strains of leishmania have been sequenced very little is known about its genes function. This is due to the absence of an RNAi pathway in the parasite, thus allowing gene function studies only by the traditional and laborious targeted gene knock out (KO) by homologous recombination methodology (W. W. Zhang & Matlashewski, 2015). However, traditional a homologous recombination for homozygous gene knockout is very difficult to achieve in a diploid organism like Leishmania, which some of its chromosomes are even triploids and tetraploids. The advent of CRISPR/Cas9 for easier targeted genome editing can potentially expand the possibility of studying gene function in leishmania and this would then allow translational research to identify new
drug targets, and/or to create attenuated strains with multiple gene knock outs could form the basis for vaccination trials.

1.6. CRISPR/Cas9 system

The Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) - Cas9 system is a new powerful approach for genome engineering in mammalian cells, insects, plants and other organisms. CRISPRs were first discovered in 1993 by the Spanish microbiologist Francisco JM Mojica at University of Alicante in Spain. He suggested that CRISPRs were used as a defense mechanism by bacteria against invading bacteriophages. In January 2013, Feng Zhang, at the Broad Institute and MIT, published for the first time his research on the use of the CRISPR/Cas9 system for genome editing in mouse and human cells. The CRISPR/Cas9 technology can be used for creating gene knock-outs, full or partial deletions, targeted insertions; even approaches for endogeneous gene activation have been described. The native CRISPR/Cas9 system in Streptococcus pyogenes consists of two short RNA molecules, CRISPR RNA (crRNA) and trans activating RNA (tracrRNA). CRISPR RNA (crRNA) is a variant oligonucleotide, whose sequence is derived from the invading bacteriophage, while the conserved trans activating RNA (tracrRNA) is derived from the bacterial cell (Figure 1.3). The tracrRNA is essential for binding of the Cas9 endonuclease, while at the same time the crRNA hybridizes to the target genome through a short homologous region, thereby guiding the CRISPR complex to cleave the double stranded DNA.
**Figure 1.2:** Targeted genome editing with RNA-guided Cas9. Modified after Charpentier & Doudna (Oregon Health & Science University, 2014). The CRISPR/Cas9 system is composed of a chimeric guide RNA (sgRNA) which is a fusion of an invariable tracrRNA (yellow) essential for binding the Cas9 enzyme, and a variable crRNA (green) specific to the targeted sequence. Cas9 endonuclease (purple) is guided by this sgRNA to the targeted gene, recognizing a PAM sequence indicated as NGG (red), and generating a double stranded break in the DNA double helix 3 nucleotides upstream of the PAM sequence. Desired editing at the DSB site is then created based on the DNA repair mechanism used by the cell.

To mimic this natural system for genome editing in other organisms, an in vitro CRISPR/Cas9 system has been designed. The system is composed of the Cas9 endonuclease enzyme and a single guide RNA molecule (sgRNA). The sgRNA bind to the nuclease enzyme and lead it to create a double stranded DNA break (DSB) at the targeted location. The sgRNA is a chimeric RNA molecule, composed of a variable 20-nucleotide crRNA that is created specific to a targeted DNA sequence and fused to a conserved 82 nucleotide tracrRNA sequence. The 20-nucleotide guide RNA is followed by a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9 enzyme. The canonical PAM sequence is 5'-NGG-3', where N can be any nucleotide. Note that this sequence will differ depending on the bacterial origin of the Cas9 enzyme.
The dsDNA cleavage is usually generated 3 bp upstream the PAM sequence. The DSB is then repaired either through an error prone repair mechanism called (Non-homology end joining (NHEJ), that results in random InDels rendering the gene non-functional, or through an error free repair mechanism called Homology direct repair (HDR), that results in faithful repair of the DNA damage.

1.7. Repair mechanisms of the double strand break generated by Cas9 endonuclease

Non-homologous end joining (NHEJ) is the principle means by which original CRISPR/Cas9 double strand breaks are repaired because it is the most prevalent type of DNA repair approach that is active at all times throughout cell cycle. It takes much less time than Homology Directed Repair (HDR) as it functions without the presence of a repair template such as a sister chromatid or other DNA homologue. Therefore, in the simple CRISPR/Cas9 system, a DSB at a targeted site is repaired by NHEJ resulting in small insertions or deletions (INDELs) that causes gene knock out (gene product loss of function).

Another mechanism for DNA repair is Microhomology-Mediated End Joining (MMEJ), where small (5-25) flanking microhomology regions contribute to defining the location
where the broken ends will be ligated. In this context, MMEJ is not as precise as HDR, yet it is much more predictable and protective than NHEJ. A study by (W. W. Zhang & Matlashewski, 2015) suggested that HDR is the dominant repair mechanism in leishmania, with MMEJ occurring in fewer instances.

1.8. Ornithine Decarboxylase: gene of choice

Since this study aim to provide a proof of concept by showing the ability to use CRISPR/Cas9 system for studying gene function, we chose a leishmanial gene that has been relatively extensively studied in the literature. The Odc gene has been amplified, cloned and sequenced for the first time in 1992 by (Hanson, Adelman, & Ullman, 1992), while its function was extensively studied using traditional gene replacement approaches in vivo and invitro as discussed below. In this context, Odc gene is suitable for testing and adapting the new strategy of gene knock out using CRISPR/Cas9 approach and where it is simply required to achieve similar results as in the traditional gene knock out approach. Odc is a gene that has been studied relatively extensively by traditional targeted gene knock out. The Ornithine Decarboxylase enzyme is a critical component of
the polyamine synthesis pathway in leishmania. This protein contributes to the production of polyamines (putrescine and spermidine) that are essential for the survival of the parasite as well as its ability to fight the immune system by inhibiting the production of NO. Polyamine synthesis pathway starts with arginine being converted into L-ornithine, after which ODC converts L. ornithine into putrescine, which in turn is converted into spermidine through spermidine synthase (SpdS) (Figure 1.4). Leishmania, like mammalian cells are auxotrophs for arginine, in which case the intracellular parasite will compete with its host macrophage cell on this amino acid to use it in polyamine synthesis. Polyamines are positively charged compounds that interact with DNA and RNA directly, and therefore they play a crucial role in regulating cell proliferation and differentiation. Furthermore, polyamines are also involved in other macromolecular production such as thiol trypanothione (Colotti & Ilari, 2011) which is essential for the parasite survival once it reaches the blood stream of the human host. Thiol trypanothione is a molecule specific to trypanosomatides, including leishmania, and is composed of two glutathione molecules joined by a spermidine polyamine linker. The major function of thiol trypanothione is defense against oxidative stress applied by the human immune system in an effort to eliminate the infective parasite. Once the leishmania promastigotes reach the blood stream of the human victim, an immune response is generated. An important aspect of this immune response is the production of nitric oxide (NO) by a variety of immune cells that help eradication of the infecting leishmania. NO is a free radical with anti-microbial activity against a wide range of bacteria and intracellular parasites including leishmania. The free radical NO is synthesized by NO synthase, by converting L-arginine into L-citrulline. NO along with superoxide (O2-) forms peroxynitrite (ONOO-), which is a potent oxidizing agent that can react with a number of different molecules including DNA, proteins and lipids leading to their severe damage and resulting cytotoxic effects (Habib & Ali, 2011). However, leishmanial parasites can inhibit the production of NO via multiple pathways, allowing them to escape the immune system and establish an infection. Also, when a parasite enters a macrophage, it forms a parasitophorous vacuole, a structure produced by parasites of the apicomplexan phylum of parasites including Leishmania in the cells of the host, that allows the parasite to develop while protected from lysosomes and NO effects. In this context, it is not a
surprise that inhibiting nitric oxide species production by thiol trypanothione in leishmania is a major defense mechanism which determine if the parasite will be able to establish an infection or will be cleared out from the system (Colotti & Ilari, 2011). A targeted gene replacement study (Jiang et al., 1999) showed that a homozygous Aodc L.donovani strain exhibits polyamine auxotrophy, a situation in which promastigotes cannot survive in a culture that lacks polyamines. The study also showed that this phenotype can be rescued by the addition of the readily available polyamines putrescine or spermidine to the medium. Hence, this study further suggested that the Odc gene is essential for parasite survival. An in vivo study (Boitz et al., 2009) showed that a homozygous Aodc strain of L.donovani is able to scavenge polyamines from the surrounding medium yet only at levels that do not allow it to sustain infection in the animal model. Ornithine Decarboxylase enzyme is thought to be a potential drug target for L.donovani and L.infantum. In fact, a-difluoromethylornithine (DFMO), a fluorinated ornithine analogue, is used for the treatment of African sleeping sickness caused by the parasitic kinetoplastid Trypanosoma brucei by irreversibly inhibiting ODC enzyme (Colotti & Ilari, 2011).

In this context, the odc gene is an essential gene for leishmania survival and infectivity, and is potential drug target. In this study, we attempt to generate Odc gene KO by interrupting the open reading frame using CRISPR/Cas9 system. The goal of this study, is to introduce and optimize CRIPR/Cas9 to L.donovani for gene editing in order to facilitate future gene function studies in this parasites.
2.1. Leishmania strain and culture medium

The *Leishmania donovani* 1S strain (MHOM/SD/62/1S), NR-48821, used in this study was obtained from BEI Resources, NIAID, NIH. Schneider's Drosophila Medium supplemented with 10% fetal calf serum, 10% sterile-filtered human male urine and 40 ug/ml gentamicin was used for routine culturing of promastigotes at 26°C. Cultures were passaged to fresh medium in a 1:50 dilution every 3-4 days.

2.2. Genomic DNA extraction, target gene sequence confirmation using PCR and Sanger Sequencing analysis

Genomic DNA was extracted from wild type (WT) *L. donovani* promastigotes. Parasites were grown into mid log-phase, a total of 15 x 10^6 cells were pelleted, and DN extraction proceeded using the Qiagen DNeasy Blood & Tissue Kit according to the manufacturer's instructions. The genomic sequence of the targeted Odc gene was retrieved from the literature and primer sets for PCR amplification were designed accordingly (ODCF1: CTCTGCATTCCGCACAGACC, ODCF2: CAAG GAAGAAATAC ATATGGTGC, ODCR1: ACTGATCTT TTCCATCTCC AGC, ODCR2: GGACGA GCACACCCCTA CAGC), where (ODCF1, ODCR1) target the 5' - half of the gene, (ODCF2, ODCR2) target the 3' - half of the gene and (ODCF1, ODCR2) target the whole 2kb gene sequence. PCR Using Q5® High-Fidelity DNA Polymerase (M0491) from was performed according to the following conditions: 30 sec initial denaturation at 98oC; 25 cycles of denaturation (10 sec at 98 °C), annealing (30 sec. at 65°C), and extension (1 min. at 72 °C); a final extension at 72 °C for 2 min. PCR amplification products were visualized on a 0.7% agarose gel electrophoresis for quality control. The full length amplified gene fragment was gel purified using the QIAquick Gel Extraction Kit, and sent for sequencing at the Biology department at Montclair State University. The same procedures were used to verify deletions generated in the gene after electroporation with both pLPhygCAS9 and pSPneogRNAH plasmids and selection.
2.3. Plasmid constructs

Plasmid constructs pLPhygCAS9 and pSPneogRNAH were a gift from Greg Matlashewski (Addgene plasmid # 63555 and # 63556 respectively). pLPhygCAS9 expresses humanized Cas9 enzyme with a nuclear localization signal and contains a Hygromycin resistance gene as selection marker. The pSPneogRNAH plasmid contains the _L. donovani_ rRNA promoter followed by the guide RNA sequence insertion sites (BbsI) and the tracrRNA sequence, followed by the HDV ribozyme coding sequence which self-cleaves the gRNA at its 3' end. G418 resistance is conferred by the neomycin selection marker in this plasmid when expressed in the parasite.

2.4. crRNA and primer design and synthesis

The crRNAs were designed manually and with the crRNA designer tool (http://www.e-crisp.org/E-CRISP/), then verified for significant on and off target matches within the _L. donovani_ genome via (http://protists.ensembl.org/Leishmania_donovani_bpk282a1/Tools/Blast?db=core).

The primers for PCR were designed manually, synthesized and purchased from Invitrogen.

2.5. Cloning guide RNA sequences into various gRNA expression vectors

The complementary single stranded oligonucleotides for crRNA were annealed in 1X Buffer 3 (New England BioLabs), by heating the mix to 95 °C for 4 min, followed by cooling down back to 20°C at a rate of 0.1°C per second using a thermocycler. Annealed oligonucleotides form the necessary BbsI overhangs so no restriction digestion is necessary for the oligoes, but only the plasmids will be digested with BbsI. pSPneogRNAH plasmid DNA was digested with BbsI restriction enzyme at 37 °C for 1 hour. Digestion was validated on a 0.7% agarose gel electrophoresis. Digested plasmid was then gel purified using the QIAquick Gel Extraction Kit. The various annealed crRNA oligonucleotides were cloned into the digested and purified plasmid in the presence of T4 DNA ligase, purchased from New England BioLabs, at 16°C for 4 hours. Recombination products were transformed into One-Shot MAX Efficiency DH5-T1R Competent Cells purchased from Invitrogen.
2.6. Verification of crRNA insertion and clonal amplification

Three clones were chosen from each ligation. Transformed cells were grown overnight in 4ml LB broth containing carbenicillin. Plasmid mini-preps were performed for each of the 21 cultures using the QIAprep Spin Miniprep Kit. The presence of an insert was verified by: first, PCR amplification using reverse primer (pSPneogRNAH R= GTGACAACGTGAGCAGCAGC) and the forward single stranded crRNA relevant to each construct as a forward primer. Also, Sanger Sequencing was performed to verify the exact crRNA insert sequence in each clone.

2.7. Leishmania Transfection and selection.

A two- plasmid strategy was used in this study one plasmid that carries the Cas9 encoding gene (pLPhygCAS9), and one plasmid carries the gRNA encoding oligos (pSPneogRNAH). First, WT *L. donovani* was transfected with pLPhygCAS9 as described in (Robinson & Beverley, 2003). Briefly, promastigotes were grown to mid-log phase. Cells were pelleted at 1300 × g for 10 min and washed in half of the original volume with electroporation buffer (EB) (120 mM KCl2, 0.15 mM CaCl2 ,10 mM K2HPO4, 25 mM HEPES, 2 mM EDTA, and MgCl2; pH 7.6). Cells were pelleted again at 1300 × g for 10 min. The pellet was resuspended in EB to a final concentration of 2 × 10^8 cells/ml. 10 ug of plasmid construct was aliquoted into a 4-mm gap cuvette. 500 ul of cells were added to the cuvette and mixed. Using a Biorad GenePulser Xcell electroporator the cells were electroporated twice at 25 uF, 1500 V (3.75 kV/cm), pausing 10 s between pulses. Cells were incubated at 26°C for 48 hrs. to recover without antibiotic pressure. Selection started 48 hrs post electroporation for three weeks in culture medium containing 100 ug/ml hygromycin. The created Cas9 expressing *L. donovani* strain was subsequently transfected with various combinations of gRNA expressing constructs, selected with G418 (100 ug/ml) for 3-4 weeks and maintained continuously in medium containing both hygromycin and G418 (100 ug/ml each).
2.8. Western Blot Analysis.

Western blot was performed to confirm the constitutive expression of Cas9 in the selected promastigotes. At mid-log phase of growth, cells were collected and lysed using Bacterial Protein Extraction Reagent (B-PER) with proteinase inhibitor. Lysed cells were centrifuged, and samples were taken from supernatant. 40 x10⁶ cells/well was used as starting material. Anti-flag primary antibody raised in mice, AP-linked anti-mouse secondary antibody and AP-chromogenic substrate were used for detection. An anti a-tubulin primary antibody was used to detect actin as a loading control.

2.9. Selection on Chemically defined medium

The polyamine deficient, chemically defined medium DMEL-CS was prepared according to (Iovannisci & Ullman, 1983), with replacing Bovine serum albumin with Chicken Serum (Table 2.1). Polyamine spermidine was added when indicated to rescue *L. donovani* promastigotes with an *Odc* gene KO.

Table 2.1. Composition of DMEL-CS

<table>
<thead>
<tr>
<th>Components*</th>
<th>Amount per liter</th>
<th>Ph</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s Modified Eagle powder (DME)</td>
<td>13.45 g</td>
<td></td>
</tr>
<tr>
<td>NaHCO3</td>
<td>3.7 g</td>
<td></td>
</tr>
<tr>
<td>Tween-80</td>
<td>40 mg</td>
<td></td>
</tr>
<tr>
<td>Hemin</td>
<td>5 mg</td>
<td></td>
</tr>
<tr>
<td>Biotin</td>
<td>1 mg</td>
<td></td>
</tr>
<tr>
<td>Chicken Serum</td>
<td>10% volume</td>
<td></td>
</tr>
<tr>
<td>Xanthine</td>
<td>0.1 Mm</td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td>10 g</td>
<td>7.0</td>
</tr>
</tbody>
</table>

*All components were pre-prepared as sterile concentrated stocks, and stored at 4°C.
CHAPTER THREE

RESULTS

3.1. Growth Characteristics of *Leishmania donovani* in the complete complex Schneider’s Drosophila medium

The use of our complete Schneider’s Drosophila medium enabled a rapid exponential promastigote growth that peaked at day 4 when the culture density reached \(\sim 9\times10^6\) Cells/ml (figure 3.1). Cultured promastigotes displayed healthy and expected characteristics as referred to in the literature: during log phase of growth, healthy promastigotes looked flagellated, had a drop-like shape, were motile, and cells undergoing binary fission were abundant. At the stationary phase cells showed a more elongated body shape, became less motile, no multiplying cells were seen and a lot of debris resulting from cell death was found in the medium. This medium is not typically used in the literature for culturing Leishmania species, but was developed through this study in our lab. The medium showed better quality and consistency in growing leishmaia promastigotes than the complete M199 medium described in the literature under our laboratory’s conditions (data not shown).

![Graph of L.donovani growth curve](image1)

![Graph of OD vs Cell count in Log phase growth](image2)

Figure 3.1. *L*. *donovani* Growth Curve in Complete Schneider’s Drosophila Medium.
3.2. The Sequence of the Odc gene from our lab L. donovani strain is identical to the sequence from the literature and databases

According to the TriTryp DB for Kinetoplastid Genomic Resources, ornithine decarboxylase in the L. donovani BPK282A1 reference strain is located on chromosome 12 (LdBPK_120105.1) as a single copy gene. The gene consists of one exon, 2124 bp in length, encoding an ODC protein that is 707 amino acids in length. The genomic reference sequence of Odc was obtained from Genbank (entry M81192). 4 primers were designed to target this sequence (ODCF1, ODCF2, ODCR1 AND ODCR2); where (ODCF1, ODCR1) amplify the first half of the gene, (ODCF2, ODCR2) amplify the second half of the gene and (ODCF1, ODCR2) amplify the whole 2kb gene sequence. PCR amplification resulted in sharp clean bands with the expected sizes, indicating that the target gene is indeed a part of our lab’s L. donovani strain. Sanger sequencing results covered ~80% of our amplified gene, showing an exact match throughout the sequenced nucleotides.

Figure 3.2. PCR amplification product of Odc gene yielded clean bands with the expected sizes molecular weight. A. Whole genomic DNA extract form L. donovani visualized on 0.7% agarose gel electrophoresis showing intact genomic DNA with no degradation. B. PCR amplification products showing clean single bands at ~2kb for DNA sample amplified using the primers pair (ODCF1 and ODCR2, lane 1) targeting the full length of Odc gene. Single band resulting from amplification of the first half of Odc using primer pair (ODCF1 and ODCR1, Lane 2), Single band resulting from amplification of the second half of Odc using primer pair (ODCF2
and ODCR2, Lane 3). 1kb DNA ladder purchased from New England BioLabs was used in each experiment (MM=Molecular Marker).

3.3. Establishing a stable *L. donovani* strain that expresses Cas9 endonuclease constitutively

In order to facilitate CRISPR/Cas9, a stable cell line has been established by episomal expression of Cas9 expressed off the plasmid pLPhygCAS9. After electroporation, cells were left to recover for 48 hours, at which time point selection started by addition of 50 and/or 100 ug/ml of hygromycin. After growing cells under antibiotic pressure for 3 consecutive weeks, western blot analysis was done to verify the expression of Cas9 endonuclease in the selected pool of promastigotes.

Figure 3.4. Western blot analysis showing expression of Cas9. Lane 1 indicate a molecular weight marker. Lanes 2 and 3 are wild type samples showing no Cas9 expression, lanes 4 and 5 are derived from cultures selected on 100 ug/ml of hygromycin for 26 days, lanes 6 and 7 are derived from cultures selected for on 50 ug/ml of hygromycin for 21 days. Lanes 2, 4 and 6 contain lysate equivalent to 20x10^6 cells as starting material, whereas lanes 3, 5 and 7 contain lysate equivalent to 40x10^6 cells as starting material.
3.4. Cloning the various crRNAs into pSPneogRNAH vectors and verification of correct insertion.

Correct insertion of each crRNA was verified by PCR using reverse primer (pSPneogRNAH R= GTGACAACGTCGAGCACAGC) and the forward single stranded crRNA relevant to each construct as a forward primer. Also, Sanger sequencing was used for further verifying the exact crRNA insert sequence in each clone (data not shown).

3.5. No InDels generated by CAS9 cleavage throughout the Odc gene

Genomic DNA from each sample as well as WT sample was extracted, and OdC gene 2kb was amplified by PCR. The amplicon was then gel purified and sent for sequencing. Sequencing analysis of Cas9 expressing *L. donovani* that were co-transfected with a sgRNA constructs showed no insertion or deletion occurring at the targeted site of Cas9 (Figure 3.5) indicating that the cleavage generated by Cas9 was repaired by Homology Directed Repair mechanism (HDR). These results are consistent with (W. W. Zhang & Matlashewski, 2015) which suggested the dominance of HDR and the absence of NHEJ in *Leishmania* as opposed to mammalian cells.
Figure 3.5: DNA sequencing analysis shows no InDels generated by CAS9 cleavage throughout the Odc gene. First line of each figure show the reference Odc sequence according to which crRNA sequences were designed, second line of each figure indicate the sequence generated after the double plasmids transfection and selection under antibiotic pressure for 3-4 weeks. For all samples. For all samples A through D, sequence analysis shows no deletion or insertion generated at the expected cleavage site by Cas9 guided by the specific indicated gRNA.
3.7. Chemically defined media DMEL-CS needs further optimization to support *L. donovani* growth.

In hypothesis, if we were capable to produce *Ode* gene knock out, *L. donovani* promastigotes will become auxotroph for polyamines putrescine and spermidine. Therefore, the KO will be lethal in the absence of readily available Polyamines in the medium. To test our hypothesis, we prepared a chemically defined medium, to which we can add or eliminate polyamines. In theory, in the absence of polyamine from this medium, wild type strain should be able to grow healthily and indefinitely, while the KO strain will not be able to survive unless spermidine or putrescine is added. Although the chemically defined medium was prepared and optimized according to literature resources, it was only able to support growth and proliferation of *L. donovani* WT for 3 consecutive passages. At first, promastigotes displayed healthy cellular characteristics including cell morphology and motility, however, a gradual decrease in doubling rate was noticed where a culture took 6 days instead of 3-4 days to reach maximum density. After the 3rd passage, the cells looked less motile and more rod-like shaped, and eventually stopped proliferating. Hence, the DMEL-CS medium as we prepared it still requires further optimization to support indefinite *L. donovani* WT strain growth before it is used for further characterization of a KO strain.
CHAPTER FOUR

DISCUSSION

Under the current worst refugee crisis in history, and as a neglected tropical disease that is most prevalent in poor countries, Leishmaniasis requires urgent development of curative medicines and vaccination. Drug and prophylaxis development in turn require more in depth knowledge about the causative agent as well as the vector transmitting the disease. For a long time, studying leishmanial gene function was very difficult due to the absence of the RNAi pathway in the parasite, making the study of gene function difficult, requiring the use of the tedious traditional approach of gene replacement by homologous recombination.

In the last decade, the CRISPR/Cas9 system has been optimized for gene knockout/knockin approaches in mammalian cells, plants and even insects. However, very little research has been done on adapting this system in studying Leishmania. Since there are core differences between applying CRISPR/Cas9 in Leishmania and mammalian cells, in this study we attempted to develop and optimize a basic CRISPR/Cas9 system in our lab for targeted gene knock out in order to facilitate future gene function studies in Lesihmanial parasite.

Over the course of this research, we were able to optimize a culture medium for growing promastigotes of *L. donovani* in our lab (Figure 3.1). The medium described here was not typically used in the cultivation of Leishmania in the literature, but rather adjusted by collaborative efforts of our lab members. This medium showed better growth characteristics, higher peak density and more consistency in growth rate and passages intervals under our laboratory’s conditions, than complete M199 mediums described in the literature. Since there is no kits available specifically for genomic DNA purification from Leishmania, we used a kit for genomic DNA extraction from human and we made necessary modifications to make compatible with DNA extraction from the parasite. With our easy modified protocol, we were able to obtain high quality and quantity of DNA that
has been validated by agarose gel electrophoresis and nanodrop. Also, cloning oligonucleotides encoding gRNAs into an expression vector, PCR and electroporation conditions were modified for serving the purpose of applying CRISPR in *L. donovani* in our lab settings.

In this study, we aimed to apply the simplest CRISPR/Cas9 system to generate a basic understanding about its adaptability to leishmania. Hence, theoretically, an introduced Cas9 in promastigotes will generate DNA double stranded breaks at the targeted site, which will then be repaired by the most prevalent repair machinery Non-Homologous End Joining (NHEJ), which will result in random insertion and/or deletion events while repairing the damage. interruption of the open reading frame with InDels would render the mutated gene product nonfunctional, and therefore the resulting phenotype can be characterized.

To facilitate the applying the above described CRISPR/Cas9 in *L. donovani*, we used two plasmids strategy where a stable promastigote cell line expressing Cas9 was created at first by shuffling in an episom carrying the Cas9 encoding gene. Hence, we were able to create a stable line of *L. donovani* promastigotes that expresses Cas9 endonuclease constitutively, expression was confirmed at 6 and 11 passages. Then, we designed multiple sgRNA constructs with which we attempted to target the Ornithine Decarboxylase gene and interrupt its open reading frame (ORF); the resulting gene knockout should facilitate a classical reverse genetic way to study its function. The next step, was transfecting the second plasmid which carries the sgRNA encoding segment and selecting tranfectants for up to four weeks under antibiotic pressure. However, DNA analysis indicated no InDel mutations occurring at the targeted site. This finding probably indicates that although Cas9 enzyme is scanning and cutting the dsDNA frequently at the targeted site, the double strand breaks are being repaired accurately each time by an error free repair machinery such as Homologous Directed Repair. These results are consistent with previous studies by (W. W. Zhang & Matlashewski, 2015) and (W. Zhang, Lypaczewski, & Matlashewski, 2017) that have reported the dominance of HDR mechanisms in the absence of Non-Homologous End Joining (NHEJ) in *Leishmania* when compared to mammalian cells. In fact, since *Leishmania* is a diploid organism, and
some of its chromosomes are even triploid and quadraploid, HDR by interchromosomal recombination is not unexpected.

Future directions for this research could include taking advantage of the HDR dominance in the parasite by co-transfecting a stop codon cassette donor oligonucleotide along with the gRNA construct. The donor in this approach would have flanking arms that are homologous to the expected Cas9 cleavage site on the DNA and therefore can be inserted into the ORF at the targeted location. Hence, the expected accurate insertion of the Stop codon cassette at the cleavage site, could potentially result in a prematurely terminated translational product that is equivalent to a gene knock out. Another approach can be targeting two sites in the gene with two specific gRNAs along with a stop codon cassette donor with flanking arms that are homologous to the expected Cas9 cleavage site where the donor can help joining the two cleaved sites and generating a deletion in the gene at the same time, thus increasing the possibility of introducing a KO mutation. Such a mutation can again result in a truncated protein that would be non-functional and thus enable the study of the phenotype resulting from this gene function by reverse genetics means.

Also, it would be important to follow up such a study with whole genome sequencing of the mutated strains, to look for possible off-target mutations that may have occurred across the genome. As has been shown in previous studies, the SpCas9 enzyme can lead to undesired off target mutations that can confound the experimental outcome. Furthermore, the Cas9 enzyme used in this study is codon optimized for use in human cells, so there could be some bias when it is being used in Leishmania due to codon translational differences between the two organisms. Cas9 variants are being used for their enhanced specificity such as Cas9 Nickase, which generates a single strand break, along with a repair template, therefore taking the most advantage of HDR in Leishmania to increase specificity.

Another difference to the mammalian system is the use of the human U6 promotor (RNA Polymerase III) to drive gRNA expression. However, this promotor has not been characterized in Leishmania, therefore, the ribosome RNA promotor (RNA
Polymerase I promotor) can be used instead in leishmania (W. W. Zhang & Matlashewski, 2015).

In summary, in this study we optimized culture medium for *L. donovani* and protocols for genomic DNA extraction as well as annealing and cloning conditions. We established a stable cell line expressing Cas9, and set up the basic strategy for applying CRISPR/Cas9 in leishmania. Overall, this study paved the way for adapting CRISPR/Cas9 system for genome editing for future proof of concept, gene function studies and identifying new drug targets and creating vaccination.
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