A SHORT INTERFERING RNA MOLECULAR BEACON FOR THE ATTENUATION OF MYCOBACTERIAL INFECTION

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ABSTRACT

The ability of the pathogen Mycobacterium Tuberculosis (MTB) to invade and survive within macrophages of granulomas is attributed to the product of the Mammalian Cell Entry (MCE) operon whose gene, mce4A, encodes a cholesterol transporter that transports host lipids into the bacterium that allows the bacterium to survive during chronic infection. Here, we proposed and tested the hypothesis that a mce4A siRNA molecular beacon can be used to attenuate mycobacterial infection in macrophages. Mce4A gene was cloned and expressed in E. coli (E. coli-4A) and differentiated U937 cells were transduced with piLenti-siRNA-GFP phage expressing the mce4A siRNA for 24 h. This was followed by infection with either E. coli-4A or M. smegmatis for 3 h followed by incubation for 0, 3, 6, 24 and 48 h. The cells were lysed and the lysates were plated on LB agar plates containing ampicillin (100 µg mL⁻¹) or on 7H11 media and incubated at 37°C overnight. Our results showed that the siRNA treatment attenuated E.coli-4A infection in macrophages at 3, 6, 24 and 48 h by 0, 77, 59.6 and 99.7%, respectively. Our results also showed that the siRNA treatment attenuated M. smegmatis infection in macrophages at 3, 6, 24 and 48 h by 94.8, 70.3, 98.9 and 93.4%, respectively. In conclusion, a mce4A siRNA molecular beacon was successfully delivered and stably expressed in macrophages which attenuated E. coli expressing mce4A (E. coli-4A) and M. smegmatis infection in macrophages.

1. INTRODUCTION

Latent Tuberculosis (LTB) is a persistent problem in both highly industrialized and developing countries (WHO, 2013; Shea et al., 2014). LTB is characterized by pulmonary granulomas which allow Mycobacterium tuberculosis (Mtb) to survive for years without detection (Barry et al., 2009; Rajni and Meena, 2011). People with LTB are unaware of their condition until their infection becomes acute in about 10-15% of cases (Mariano, 1995). In the U.S. alone, more than 80% of Tuberculosis (TB) cases are from reactivation of LTB infection (Horsburgh and Rubin, 2011). The hallmark of LTB is the granulomas harboring the bacterial infection along with their draining lymph nodes. These granulomas have been shown to possess a necrotic core in the center that provides nutritional source for the persisting Mtb bacteria and is surrounded by concentric layers of macrophages, epitheloid cells, multinucleated Langhans giant cells and lymphocytes (Mariano, 1995; Parasa et al., 2013). These TB lesions are surrounded by highly vascularized tissue (Ulrichs et al., 2005) which enables the targeting of...
Latent Mtb with systemically administered drugs. Cases of LTB are on the rise in industrialized nations like U.S owing to the increased transnational migration of populations (Walter et al., 2014). Because of its asymptomatic nature, LTB is difficult to treat (Horsburgh and Rubin, 2011). The treatment regimen for both active and LTB infection consists of an extended course of antibiotics like isoniazid or rifampicin spanning many months which generally carries poor patient compliance rates (Horsburgh and Rubin, 2011). Therefore, because there is lack of efficient treatment for LTB, this study was conducted in order to design a siRNA molecular beacon against one of the mammalian cell entry protein genes that could be used for the attenuation of mycobacterial infection in macrophages.

*M. tuberculosis* survival in granulomas is made possible through its ability to synthesize a set of mammalian cell entry proteins, *MCE4* (Arruda et al., 1993). These proteins are encoded on the mce4 operon that consists of five genes designated *mce4A*-F. During survival of *M. tuberculosis* in the macrophage, these genes are specifically expressed and bioinformatics studies have shown that the products of these genes are cholesterol transporters that help transport lipids from the host macrophage into the mycobacterium allowing the mycobacterium to survive for years during chronic infections (Pandey and Sassetti, 2008). The mce operons are widely distributed throughout the genus Mycobacterium and a non-pathogenic mycobacterial species *M. smegmatis*, that shares many features with *M. tuberculosis*, possesses a homolog of *mce4* (Altschul et al., 1990; Haile et al., 2002; Rathor et al., 2013). Owing to its lower biosafety level restrictions and the presence of an identical *MCE4* cholesterol transport system, *M. smegmatis* (Ms) provides a safe mycobacterial model for preliminary studies (Pelosi et al., 2012).

Since about 90% of the TB patients develop LTb and 10% go on to have acute TB (Kumar and Robbins, 2012), prompt diagnosis and treatment of individuals with LTB is important for the effective control of this disease. Thus, developing a direct Mtb treatment strategy for the asymptomatic latent TB population is vital to our fight against tuberculosis.

Molecular Beacon (MB) siRNA is hairpin shaped single stranded antisense nucleic acid construct with a stem-loop structure. It is postulated that the siRNA stimulates a yet unknown cell surface molecule that initiates the taking up of the siRNA into the host cell via caveoae into caveosomes, which is then transported to the perinuclearly located smooth ER and finally released into the host cell cytoplasm (Erdmann and Barciszewski, 2010). The siRNA is taken up by the bacteria within the host cell and in the presence of the target *mce4A* mRNA, the loop region of the molecular beacon siRNA hybridizes with the target mRNA. The siRNA-mRNA duplex induces the bacterial interference machinery to cleave the target mRNA and releases the fragments (Van Der Oost and Brouns, 2009; Wiedenheft et al., 2012). The degradation of the *mce4* mRNA ultimately results in depriving the mycobacterium of its energy and carbon sources leading to its loss of virulence and death (Pandey and Sassetti, 2008; Miner et al., 2009; Van Der Geize et al., 2007).

Latent TB is a silent epidemic that threatens the development and fundamental progress of many societies across the globe. In order to counteract this epidemic, effective treatment for TB is critical. To this end, we tested the hypothesis that *M. smegmatis* infection can be attenuated in macrophages using the siRNA molecular beacon against the *mce4A* operon gene that we have previously designed and tested (George et al., 2012). The findings of these studies will demonstrate the utility of attenuating mycobacterial infection using siRNA molecular beacons which can then be easily adapted to eradicating infection in animal models and eventually in humans.

### 2. MATERIALS AND METHODS

#### 2.1. Mammalian Cell Culture

U937 human monocytic cells were maintained in RPMI medium supplemented with 10% fetal calf serum and 50 U mL⁻¹ of penicillin and streptomycin in a 5% CO₂ humidified atmosphere. Cells were passaged at a density of approximately 2×10⁶ cells mL⁻¹ every other day. Cells were cultured in flasks at 37°C for propagation and in 12 well plates with rat-tail collagen coated glass cover slips for differentiation and infection. U937 cell differentiation was achieved as described previously (Adunyah et al., 1992). Briefly, cells were counted using a hemocytometer and 1×10⁶ cells were incubated with complete RPMI medium containing 4nM PMA. After 48 h of treatment with PMA, nonadherent cells were aspirated and fresh media was added prior to initiating treatment.

#### 2.2. Bacterial Strains and Culture Conditions

*M. smegmatis* mc² 155 was purchased from ATCC and grown to log phase *A₅₀₀* of 0.3 in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween 80 and
OADC (oleic acid, albumin, glucose, catalase supplement) in 37°C with constant shaking at 190rpm. Serial dilutions of the culture were prepared at 1:10, 1:100 and 1:000 in 7H9 media, plated on 7H11 agar plates and incubated at 37°C. Colonies were counted and the number of Colony Forming Units (CFUs) was determined to calculate the Multiplicity of Infection (MOI) for invasion assay experiments.

E. coli-4A was generated by transforming E. coli TOP10® (Invitrogen) with pTrcHis2-TOPO (Invitrogen) containing the mce4A gene from M. smegmatis. E. coli-4A clones were grown on Lennox Broth (LB) containing 100μg mL\(^{-1}\) ampicillin and the expression of the recombinant protein was induced by adding Isopropyl-β-D-thio-Galactoside (IPTG) to the log-phase cultures at a final concentration of 1mM and grown at 37°C with shaking for 5 h. Serial dilutions of the culture were prepared at 1:10, 1:100 and 1:000 in LB media, plated on LB agar plates and incubated at 37°C. Colonies were counted and the number of Colony Forming Units (CFUs) was determined to calculate the Multiplicity of Infection (MOI) for invasion assay experiments.

2.3. Cloning of siRNA in piLenti-GFP

The siRNA target sequence for mce4A gene was selected using antisense design software provided by Integrated DNA Technologies (IDT). A region of the target sequence spanning nucleotides 5960719-5960747 of M. smegmatis coding region (5’-TCGGCAGGCTTCGGGATAGGTGTATCCC-3’) was identified and 4 nucleotides were added to 5’ end of each strand in order to facilitate the cloning of this fragment into Bbs I site of the iLenti-GFP vector (Applied Biological Materials, Inc.). This sequence was designed so that upon transcription, the transcript will form a hairpin structure. This double stranded (ds) fragment was cloned into the iLenti-GFP vector by mixing an aliquot (3μL) of the ds sequence, 4μL of Bbs I linearized piLenti-GFP vector, 2μL of 5xDNA ligase buffer and 1μL of T4 DNA ligase followed by incubation at room temperature for 2 h. The ligation mix was used to transform E. coli DH5α cells followed by plating on LB agar plates containing 50μg mL\(^{-1}\) kanamycin and overnight incubation at 37°C. Ten colonies were selected and grown overnight in LB broth containing 50μg mL\(^{-1}\) kanamycin. Plasmid isolation from each of the colonies was performed using the SNAP Midiprep kit (Invitrogen) according to the manufacturer’s instructions and the plasmids were screened by EcoRV digestion to identify recombinant clones with the correct size and orientation of the insert. E. coli containing the insert in the correct orientation was used for lentivirus production.

2.4. Generation of piLenti-siRNA-GFP Phage

The piLenti-siRNA-GFP phage was generated in 293T cells. 293T cells were plated in a 10cm tissue culture plate at 90% confluency. A transfection mix was premade in 2mL complete medium supplemented with 0.1mM MEM Non-Essential Amino Acids, 4mM L-Glutamine, 1mM sodium pyruvate, 500 μg mL\(^{-1}\) Geneticin, along with 10μg piLenti-siRNA-GFP vector, 10μg packaging plasmids (Lenti-Combo Mix®, Applied Biological Materials, Inc.) and 80μL of lentifectin® transfection reagent (Applied Biological Materials, Inc.), followed by incubation at room temperature for 20 min and addition of an extra 4.5mL serum-free medium. 293T cells were transfected with the transfection mix followed by incubation at 37°C in a humidified 5% CO\(_2\) incubator. Viral supernatants were harvested after 48 h of transfection and filtered through 0.45-μm PVDF syringe filter (Millipore) followed by concentration using Ultra-Pure® (Applied Biological Materials, Inc.) lentivirus purification kit. Viral titers were determined by RT-PCR using Lentiviral qPCR Titre Kit (Applied Biological Materials, Inc.) according to the manufacturer’s instructions.

2.5. Immunofluorescence

U937 cells were differentiated with 4nM PMA for 48 h in a 12 well plate containing rattle collagen coated coverslips at 1×10\(^{5}\) cells per well. Seeded cells were either transduced or not transduced with piLenti-siRNA-GFP construct at a MOI of 5:1 for 24 h. Cells were washed in 1xPBS, fixed for 15 min with 4% Paraformaldehyde (PFA) and mounted on slides using Vectashield® medium containing propidium iodide. Fluorescence imaging was accomplished using a confocal microscope (inverted Nikon TE2000-U microscope equipped with a 60×apochromat oil-immersion TIRFM objective).

2.6. Western Blot

Western blot analysis was performed on lysates from differentiated U937 cells which were transduced with the piLenti-siRNA-GFP phage for 24 h, followed by infection with either E. coli-4A or M. smegmatis for 3 h followed by incubation for 0, 3, 6, 24 and 48 h. The cells were washed, lysed and the lysate was used...
for Western blot analysis using a GFP monoclonal antibody as previously described (Unlap and Jope, 1997). Specifically, 5.2 µg of each protein extract was diluted with Laemmli sample buffer, placed in a boiling water bath for 5 min and electrophoresed on 10% SDS-polyacrylamide gel followed by electrotransferring for one hour at 100V. Subsequent to Western blotting, the nitrocellulose membrane (Pierce) was rinsed in 10 mL Phosphate Buffered Saline (PBS), pH 7.4. It was then blocked using 10 mL of Blotto (PBS/5% low-fat dried milk/0.1% Tween 20), at 4°C with slow shaking for 3 h. Following rinsing with PBS the membrane was slowly shaken overnight with a 1:7500 dilution of rabbit polyclonal anti-GFP antibody (Invitrogen, Inc). The blot was rinsed twice with PBS/0.1% Tween 20 and then washed three times for 5 min each using 100 mL volumes of PBS/0.1% Tween 20. The membrane was probed for 2 h at 4°C with a 1:5000 dilution of Horseradish Peroxidase (HRP) conjugated goat anti-rabbit IgG (Invitrogen) in 10 mL Blotto. Visualization of GFP protein was achieved by the use of an ECL kit (Pierce) rinsed in 10 mL Phosphate Buffered Saline (PBS), pH 7.4. It was then blocked using 10 mL of Blotto (PBS/5% low-fat dried milk/0.1% Tween 20), at 4°C with slow shaking for 3 h. Following rinsing with PBS the membrane was slowly shaken overnight with a 1:7500 dilution of rabbit polyclonal anti-GFP antibody (Invitrogen, Inc). The blot was rinsed twice with PBS/0.1% Tween 20 and then washed three times for 5 min each using 100 mL volumes of PBS/0.1% Tween 20. The membrane was probed for 2 h at 4°C with a 1:5000 dilution of Horseradish Peroxidase (HRP) conjugated goat anti-rabbit IgG (Invitrogen) in 10 mL Blotto. Visualization of GFP protein was achieved by the use of an ECL kit for the detection of HRP-labeled antibodies on Western blots (Fisher, Inc). The blot was placed in a film cassette and exposed to X-ray film (Hyperfilm-ECL, GE) and developed.

2.7. Invasion Assay Time-Course

PMA differentiated U937 cells were seeded at 2.5×10³ cells per well in 12-well plates and incubated for 24h. Seeded cells were either transduced or not-transduced with piLenti-siRNA-GFP construct at a MOI of 5:1 for 24 h. Transduced U937 cells were incubated in fresh medium (HyQ DMEM supplemented with 5% FCS and 2 mM L-glutamine) at 37°C for 30 min and E.coli-4A or M. smegmatis were added to each well at a MOI of 10:1 and incubated at 37°C for 3 h. Cells were washed 3 times with HyQ DMEM media which contained 5% Fetal Bovine Serum (FBS), 1% pen/strep and 100 µg mL⁻¹ kanamycin to remove extracellular bacteria and lysed after 0, 3, 6, 24 and 48h. For lysis, cells were incubated for 10 min in 500 µL of lysis buffer (0.1% Triton X-100 in PBS, pH 7.4) and the lysate was plated on LB agar plates containing ampicillin (100µg mL⁻¹) or on 7H11 media and incubated at 37°C overnight. Recombinant E. coli-4A or M. smegmatis colonies were counted and the numbers of colonies that survived after 0, 3, 6, 24 and 48 h post infection was plotted versus time. Infection levels at 3, 6, 24 and 48 h were compared to that of base line level (0 h) using Analysis of Variance (ANOVA).

2.8. Statistical Analysis

The effect of each treatment was compared between treated and non-treated using ANOVA. A p value ≤ 0.05 is considered significant.

3. RESULTS

3.1. Cloning of mce4A siRNA in piLenti-GFP Vector

A region of the mce4A gene in M. smegmatis MC1557 was cloned into the piLenti-GFP vector in order to generate a siRNA against the mce4 messenger RNA (Fig. 1). The cloning of the mce4A region of the gene allowed the expression of the siRNA with two complementary 5' and 3' ends that will allow the formation of a hairpin structure consisting of a loop, which is the antisense RNA sequence and a stem which is a random sequence that is complimentary between the 5’ and 3’ ends. The EGFP reporter gene incorporated under the CMV promoter of the piLenti-siRNA-GFP vector allowed the tracking of expressed siRNA in vivo. The successful cloning of the fragment was confirmed by EcoR V restriction digest.

3.2. Generation of Pilenti-siRNA-GFP Phage

The generation of the phage was accomplished by transfecting piLenti-siRNA-GFP vector along with packaging plasmids into 293T cells using lentifectin® transfection reagent (Applied Biological Materials, Inc.). Viral supernatants harvested after 48 h of transfection were filtered through 0.45 µm PVDF syringe filter, concentrated using Ultra-Pure® (Applied Biological Materials, Inc.) lentivirus purification kit and viral titers were determined by RT-PCR using Lentiviral qPCR Titre Kit (Applied Biological Materials, Inc.). The viral titer was found to be 10⁶ IU mL⁻¹.

3.3. Immunofluorescence

In order to determine the efficiency of U937 cell transduction with piLenti-siRNA-GFP phage, U937 cells were transduced with the piLenti-siRNA-GFP phage and imaged using a fluorescent microscope. The results show that cells that were not transduced with the piLenti-siRNA-GFP phage did not show any green fluorescence while cells that were transduced with the piLenti-siRNA-GFP phage showed green fluorescence (Fig. 2).
**Fig. 1.** *piLenti-siRNA-GFP* vector containing the siRNA molecular beacon construct. A 29bp region of the *M. Smegmatis* MC2155 *mce4A* gene which spans nucleotides 5960719-5960747 was cloned into *piLenti*-GFP at the BbsI site. Transcription of this fragment is driven by the U6 promoter and generates a *mce4A* siRNA.

**Fig. 2.** Transduced U937 cells express GFP. U937 macrophages were differentiated on rat tail collagen coated coverslips for 48 h with 4nM PMA and were either not transduced (A) or transduced (B) with *piLenti-siRNA-GFP* construct at a MOI of 5:1 for 24 h. Cells were washed in 1×PBS, fixed for 15 min with 4% PFA and mounted on slides using Vectashield® medium containing propidium iodide followed by fluorescence imaging using a confocal microscope.
3.4. Western Blot Analysis

To confirm the expression of the GFP protein, Western blot analysis was performed on lysates from differentiated U937 cells which were transduced with the piLenti-siRNA-GFP phage for 24 h, followed by infection with either E. coli-4A or M. smegmatis for 3 h followed by incubation for 0, 3, 6, 24 and 48 h. The cells were washed and lysed and the lysate was used for Western blot analysis using a GFP monoclonal antibody. The results show that the transfected cells constitutively expressed the GFP protein at all of the time points that were examined (Fig. 3).

3.5. Invasion Assay

After confirming the expression of the GFP protein by immunofluorescence and Western blot analyses, invasion assay was carried out to determine the effect of the mce4A siRNA on macrophage infection. Differentiated U937 macrophages were transduced with piLenti-siRNA-GFP phage for 24 h followed by infection with E.coli-4A or M. smegmatis for 3 h and incubated for 0, 3, 6, 24 and 48 h. The cells were extensively washed and lysed in 0.1% Triton-X 100 lysis buffer. The lysates were plated on either LB agar containing ampicillin (100 µg mL\(^{-1}\)) or 7H11 media for E. coli-4A or M. smegmatis, respectively. The degree of attenuation of E. coli-4A infection was compared between 3, 6, 24 and 48 h against that at 0 hr and was found to be 0, 77, 59.6 and 99.7%, respectively. The degree of attenuation of M. smegmatis infection was compared between 3, 6, 24 and 48 h against that at 0 hr and was found to be 94.8, 70.3, 98.9 and 93.4%, respectively.

Fig. 3. The GFP is immunodetected in transduced U937 cells infected with E.coli-4A or M. smegmatis. U937 macrophages were differentiated for 48 h with 4nM PMA in 12-well plates at 1×10\(^6\) cells per well and transduced with piLenti-siRNA-GFP construct at a MOI of 5:1 for 24 h. Transduced U937 cells were infected with IPTG-induced log phase cultures of recombinant E. coli-4A or M. smegmatis MC\(^2\)155 at a MOI of 10:1 for 3 h, followed by extensive washing and incubation for 0, 3, 6, 24 and 48 h. Total cell lysate was obtained at each time point, fractionated on SDS-PAGE and Western blotting was performed using a rabbit polyclonal anti-GFP antibody and HRP conjugated goat anti-rabbit antibody. GFP immunodetection in transduced U937 cells infected with E.coli-4A (A) or M. smegmatis (B) was accomplished using the Immobilon Western Chemiluminescent HRP Substrate (ECL) System® (Millipore). Molecular mass standards, EZ-Run Prestained Protein Marker® (Fisher), are indicated in k

Fig. 4. Mce4A siRNA attenuates E.coli-4A or M. smegmatis infection in U937 cells. Differentiated U937 cells were transduced at MOI of 5:1 with piLenti-siRNA-GFP lentivirus for 24 h and were infected with IPTG-induced log phase cultures of recombinant E. coli-4A or M. smegmatis MC\(^2\)155 at a MOI of 10:1 for 3 h, followed by extensive washing and incubation for 0, 3, 6, 24 and 48 h. Cells were washed extensively in 1xPBS, lysed in 0.1% Triton-X 100 lysis buffer and the lysate was plated on 7H11 agar plates or on LB agar plates containing ampicillin (100µg mL\(^{-1}\)) followed by incubation at 37°C
overnight. E. coli-4A (A) and M. smegmatis (B) colonies are counted and the numbers at 3, 6, 24 and 48 h post infection are compared with those at 0 hr using ANOVA. n = 4; ‘p<0.05; **p<0.01. Our results showed that mce4A siRNA attenuated E. coli-4A and M. smegmatis infection in macrophages and the degree of attenuation of E. coli-4A and M. smegmatis infection was found to be significant (p<0.05) (Fig. 4).

4. DISCUSSION

The use of molecular beacons and siRNAs for in vivo detection and knockdown of mRNA is gaining popularity. These small hairpin-shaped antisense ribooligonucleotides are stable in cellular environments and bind to their target mRNAs with high specificity (Rhee et al., 2008; Desai et al., 2014). They can easily be modified by the addition of fluorophores and quenchers in order to enhance their utility in detection protocols in vivo (Santangelo et al., 2006; Kim et al., 2008; Bratu et al., 2011; Xue et al., 2011; Hernandez et al., 2014).

Molecular beacon based short interfering RNA (MB siRNA) has also been proven to be a powerful tool for therapeutic gene silencing because of its specificity, broad applicability and high efficiency (Kim et al., 2008; Hong et al., 2010; Ilieva et al., 2013). Small interfering RNA (siRNA) technology has been used to inhibit transcription of hepatitis G virus (Cao et al., 2005), influenza virus (Ge et al., 2003), picorna virus (Lim et al., 2008) and trypanosoma brucei (Zhang et al., 2007). SiRNA molecular beacons have been used successfully in the detection and knockdown of telomerase expression in human breast cancer cells (Chang et al., 2007), BMP4 mRNA in hedgehog signaling (Rhee et al., 2008) and aromatase mRNA in breast cancer cells (Zhou et al., 2011). SiRNA technology has been tested successfully for imaging and silencing genes in M. tuberculosis (Harth and Horwitz, 1999; Li et al., 2007) and for inhibiting bacterial growth in human macrophages infected with M. tuberculosis (Harth et al., 2007).

The potential combination of the two technologies, molecular beacon and siRNA, in the health care industry is tremendous. We had previously shown that a siRNA molecular beacon labeled with the fluorophore TYE 665 and quencher Iowa Black RQ-SP could be used for the detection of M. smegmatis infection in macrophages (George et al., 2012).

The present study was necessitated by three factors, (1) latent TB continues to be a problem not only for the developing countries but also for industrialized nations like the U.S., (2) because the mycobacterium growth rate is so slow, rapid and specific diagnostic or imaging tests for latent TB are currently not available which naturally leads to (3) there is a lack of specific and efficient treatment for latent TB. To assist in the effort to treat latent TB, this study was conducted in order to test the hypothesis that a molecular beacon siRNA designed against the mce4 operon, which has been shown to be responsible for latent TB infection (Arruda et al., 1993; Saini et al., 2008; Rathor et al., 2013), especially mce4A (Saini et al., 2008; Xu et al., 2007), could be used for the attenuation of mycobacterial infection. This hypothesis was tested in differentiated U937 cells infected with recombinant E. coli expressing mce4A gene or M. smegmatis.

In order to design the molecular beacon siRNA, it was first necessary to determine which of the mce4 operon genes conferred the highest degree of virulence to its host. Our previous studies with recombinant E. coli expressing various mce4 operon genes showed that mce4A gene conferred the highest degree of virulence to its host E. coli (George et al., 2012). Because the M. tuberculosis has a slow growth rate and since there is high degree of homology between mce4 operons of M. tuberculosis and other mycobacteria (Haile et al., 2002), the mce4A gene of the rapid growing M. smegmatis was selected as the target for our siRNA based mycobacterial infectivity studies.

The mce4A molecular beacon antisense RNA was designed to contain a double stranded stem which consists of nucleotides that are complementary to each other to form a 5-base pair double stranded stem. The loop consists of 29 nucleotides that are complementary to a region of the target mce4A mRNA. The double stranded stem facilitates cytosolic localization of the siRNA (Chen et al., 2010) in the bacterium and in the presence of the target mce4A mRNA, the siRNA molecular beacon will bind and degradation of the target mRNA will be induced. The transduction of the molecular beacon siRNA construct using a lentiviral vector expressing GFP (Fig. 1), ensured that the siRNA was constitutively expressed within the differentiated U937 cells throughout the various experimental time points, even up to 48 h post infection (Fig. 2 and 3).

This molecular beacon design tests the rationale that in the absence of the target mce4A mRNA the molecular beacon remains in its hairpin form while in the presence of its target mRNA the 29 nucleotide loop will compete with the 5 nucleotide stem for hybridization to their respective targets, the loop to the mce4A mRNA and the stem to its complementary pair on the opposite ends of the molecular beacon. The hybridization of the loop to its target will be greater than that of the strands in the stem, based on the number of nucleotides (29 versus 5). Hybridization of the loop to the mce4A mRNA will create a temporary siRNA-mRNA duplex, which induces the bacterial RNA.
interference machinery) to cleave the target mRNA and release the fragments (Van Der Oost and Brouns, 2009; Wiedenheft et al., 2012). Because the mycobacterium utilizes the product of mce4A for survival on cholesterol for carbon and energy source (Xu et al., 2007; Senaratne et al., 2008; Miner et al., 2009; Rathor et al., 2013), degradation of the mce4A mRNA will lead to its reduced survival. This study tested the ability of the mce4A siRNA to attenuate its target mce4A mRNA in macrophages infected with recombinant E.coli-4A and M. smegmatis. The results show that the molecular beacon siRNA construct attenuates its target in macrophages infected with either E. coli-4A or M. smegmatis (Fig. 4). Thus, a molecular beacon can be designed against one of the mce4 operon genes in M. smegmatis that facilitates the eradication of mycobacterial infection in macrophages.

5. ACKNOWLEDGEMENT

We have used a GFP expressing lentiviral vector piLenti-siRNA-GFP to successfully deliver and stably express the mce4A siRNA molecular beacon construct in macrophages infected with either E. coli expressing the mce4A gene or M. smegmatis. Our results showed that the mce4A siRNA was able to attenuate E. Coli-4A and M. smegmatis infection in macrophages.

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