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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.

Keywords: Latent TB, Molecular Beacon, *Mce4*, Mycobacteria, siRNA

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

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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 caveolae 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 it 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% CO_2 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 rattail 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 mc^2 155 was purchased from ATCC and grown to log phase A_{600} 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'-TCGGCAGGCTCTCGGGATAGGTGTATCCC-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 generate a siRNA with complimentary 5' and 3' ends that 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 5×DNA 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 *EcoR V* 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 rattail 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 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 2.5×10^5 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 we*re 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% penn/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 MC155*[2] 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 10^6 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**).

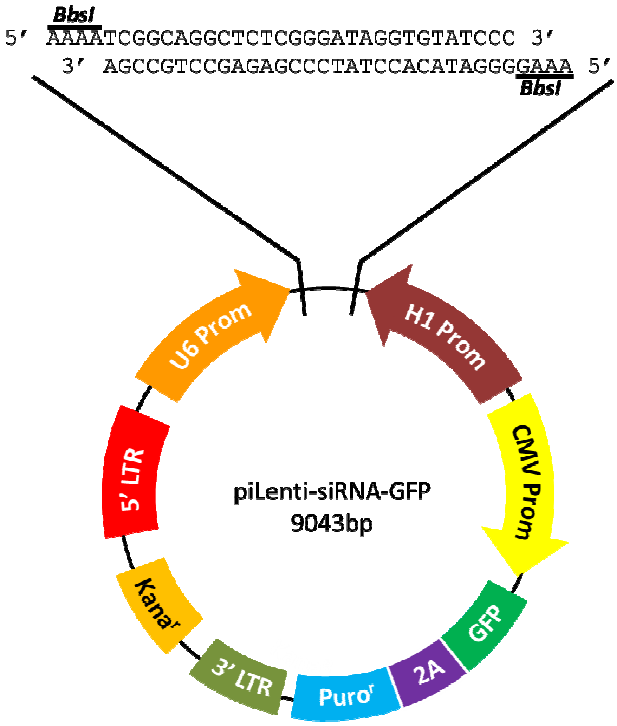
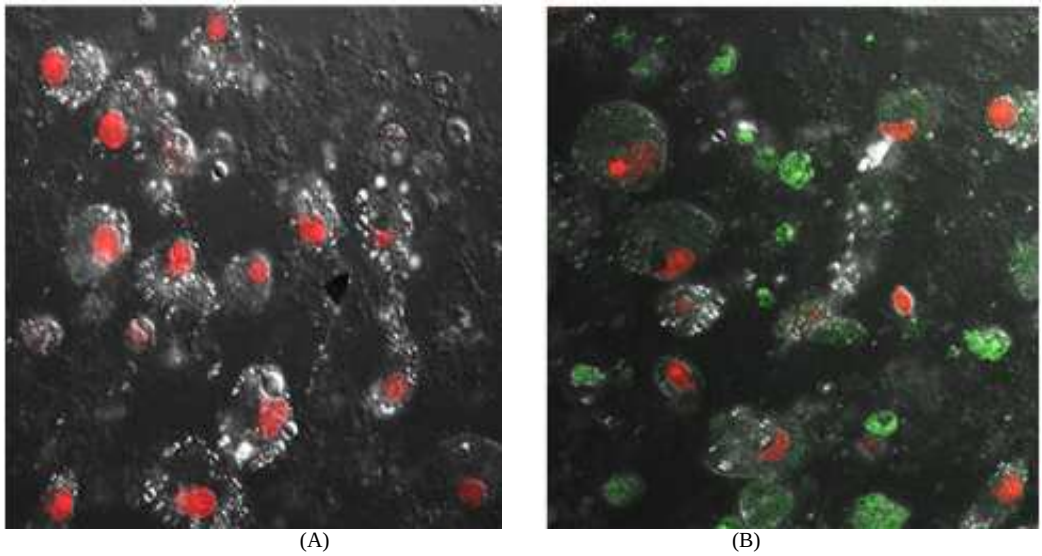


Fig. 1. *piLenti*-siRNA-*GFP* vector containing the siRNA molecular beacon construct. A 29bp region of the *M. Smegmatis* MC2155 *mce4A* gene which spans nucleotides 5960719-5960747 was cloned into piLenti-GFP at the BbsI site. Transcription of this fragment is driven by the U6 promoter and generates a *mce4A* siRNA.



(A) (B)

Fig. 2. Transduced U937 cells express GFP. U937 macrophages were differentiated on rattail 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 0hr and was found to be 94.8, 70.3, 98.9 and 93.4%, respectively.

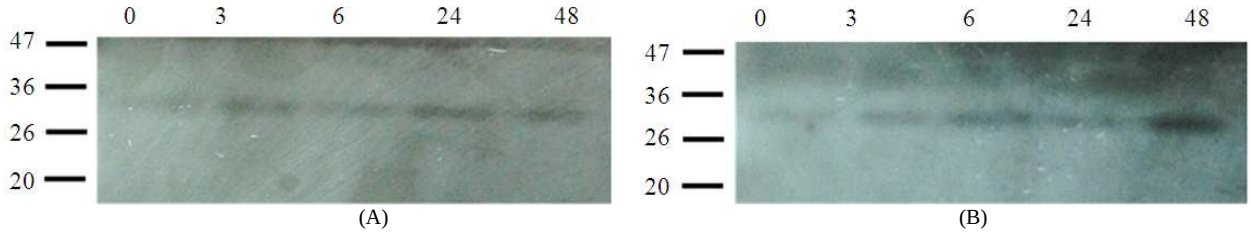


Fig. 3. The GFP is immunodetected in transduced U937 cells infected with *E.coli-4A* or *M. smegmatis*. U937 macrophages were differentiated for 48 h with 4nM PMA in 12-well plates at 1×10^6 cells per well and transduced with *piLenti*-siRNA-*GFP* construct at a MOI of 5:1 for 24 h. Transduced U937 cells were infected with IPTG-induced log phase cultures of recombinant *E. coli-4A* or *M. smegmatis MC²155* 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

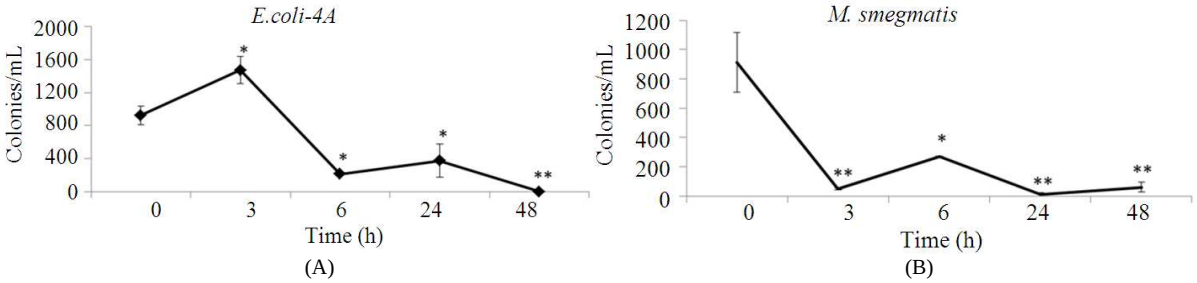


Fig. 4. *Mce4A* siRNA attenuates *E.coli-4A* or *M. smegmatis* infection in U937 cells. Differentiated U937 cells were transduced at MOI of 5:1 with *piLenti*-siRNA-*GFP* lentivirus for 24 h and were infected with IPTG-induced log phase cultures of recombinant *E. coli-4A* or *M. smegmatis MC²155* at 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 1×PBS, 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 0hr using ANOVA. n = 4; $^{*}p\leq0.05$; $^{**}p\leq0.01$

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 ($p\leq0.05$) (**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 trypanosma 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. CONCLUSION

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.

6. ACKNOWLEDGEMENT

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7. REFERENCES

Adunyah, S.E., T.M. Unlap, C.C. Franklin and A.S. Kraft, 1992. Induction of differentiation and c-jun expression in human leukemic cells by okadaic acid, an inhibitor of protein phosphatases. J. Cellular Phys., 151: 415-426. DOI: 10.1002/jcp.1041510223

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman, 1990. Basic local alignment search tool. J. Mol. Biol., 215: 403-410. DOI: 10.1016/S0022-2836(05)80360-2

Arruda, S., G. Bomfim, R. Knights, T. Huima-Byron and L.W. Riley, 1993. Cloning of an M. tuberculosis DNA fragment associated with entry and survival inside cells. Science, 261: 1454-1457. DOI: 10.1126/science.8367727

Barry, C.E., H.I. Boshoff, V. Dartois, T. Dick and S. Ehrt *et al*., 2009. The spectrum of latent tuberculosis: Rethinking the biology and intervention strategies. Nat. Rev. Microbiol., 7: 845-855. DOI: 10.1038/nrmicro2236

Bratu, D.P., I.E. Catrina and S.A. Marras, 2011. Tiny molecular beacons for *in vivo* mRNA detection. Methods Mol. Biol., 714: 141-157. DOI: 10.1007/978-1-61779-005-8_9

Cao, M., H. Ren, P. Zhao, W. Pan and L. Zhao *et al*., 2005. Small interfering RNA-mediated inhibition of hepatitis G virus gene expression in human hepatoma cell Huh-7. Sci. China C Life Sci., 48: 61-69. DOI: 10.1360/03yc0233

Chang, E., M.Q. Zhu and R. Drezek, 2007. Novel siRNA-based molecular beacons for dual imaging and therapy. Biotechnol. J., 2: 422-425. DOI: 10.1002/biot.200600257

Chen, A.K., O. Davydenko, M.A. Behlke and A. Tsourkas, 2010. Ratiometric bimolecular beacons for the sensitive detection of RNA in single living cells. Nucleic Acids Res., 38: e148-e148. DOI: 10.1093/nar/gkq436

Desai, H.V., I.S. Voruganti, C. Jayasuriya, Q. Chen and E.M. Darling, 2014. Live-cell, temporal gene expression analysis of osteogenic differentiation in adipose-derived stem cells. Tissue Eng. Part A. DOI: 10.1089/ten.TEA.2013.0761

Erdmann, V.A. and J. Barciszewski, 2010. RNA Technologies and Their Applications. 1st Edn., Springer, New York, ISBN-10: 3642121683, pp: 468.

Ge, Q., M.T. McManus, T. Nguyen, C.H. Shen and P.A. Sharp *et al*., 2003. RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. Proc. Natl. Acad. Sci. USA, 100: 2718-2723. DOI: 10.1073/pnas.0437841100

George, R., N. Bolus, J. Garner, K. Nugent and M.T. Unlap, 2012. A short interfering RNA (siRNA) molecular beacon for the detection of mycobacterial infection. J. Biotechnol. Biomaterials, 2: 1000147-1000147. DOI: 10.4172/2155-952X.1000147

Haile, Y., D.A. Caugant, G. Bjune and H.G. Wiker, 2002. *Mycobacterium tuberculosis* mammalian cell entry operon (mce) homologs in Mycobacterium Other Than Tuberculosis (MOTT). FEMS Immunol. Med. Microbiol., 33: 125-132. DOI: 10.1016/S0928-8244(02)00302-4

Harth, G. and M.A. Horwitz, 1999. An inhibitor of exported *Mycobacterium tuberculosis* glutamine synthetase selectively blocks the growth of pathogenic mycobacteria in axenic culture and in human monocytes: Extracellular proteins as potential novel drug targets. J. Exp. Med., 189: 1425-1436. DOI: 10.1084/jem.189.9.1425

Harth, G., P.C. Zamecnik, D. Tabatadze, K. Pierson and M.A. Horwitz, 2007. Hairpin extensions enhance the efficacy of mycolyl transferase-specific antisense oligonucleotides targeting *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA, 104: 7199-7204. DOI: 10.1073/pnas.0701725104

Hernandez, F.J., L. Huang, M.E. Olson, K.M. Powers and L.I. Hernandez *et al*., 2014. Noninvasive imaging of Staphylococcus aureus infections with a nuclease-activated probe. Nat. Med. DOI: 10.1038/nm.3460

Hong, H., Y. Zhang and W. Cai, 2010. *In vivo* imaging of RNA interference. J. Nucl. Med., 51: 169-172. DOI: 10.2967/jnumed.109.066878

Horsburgh, C.R. Jr. and E.J. Rubin, 2011. Clinical practice. Latent tuberculosis infection in the United States. N. Engl. J. Med., 364: 1441-1448. DOI: 10.1056/NEJMcp1005750

Ilieva, M., P. Della Vedova, O. Hansen and M. Dufva, 2013. Tracking neuronal marker expression inside living differentiating cells using molecular beacons. Front Cell Neurosci., 7: 266. Doi: 10.3389/fncel.2013.00266

Kim, Y., D. Sohn and W. Tan, 2008. Molecular beacons in biomedical detection and clinical diagnosis. Int. J. Clin. Exp. Pathol., 1: 105-116.

Kumar, V. and S.L. Robbins, 2012. Robbins Basic Pathology. 9th Edn., Philadelphia, PA. ISBN-13: 978-1437717815

Li, Y., Z. Chen, X. Li, H. Zhang and Q. Huang *et al*., 2007. Inositol-1-phosphate synthetase mRNA as a new target for antisense inhibition of *Mycobacterium tuberculosis*. J. Biotechnol., 128: 726-734. DOI: 10.1016/j.jbiotec.2006.12.019

Lim, T., J. Yuan, H.M. Zhang, A. Sall and Z. Liu *et al*., 2008. Antisense DNA and RNA agents against picornaviruses. Front Biosci., 13: 4707-4725. DOI: 10.2741/3034

Mariano, M., 1995. The experimental granuloma. A hypothesis to explain the persistence of the lesion. Rev. Inst. Med. Trop. Sao Paulo, 37: 161-176. DOI: 10.1590/S0036-46651995000200012

Miner, M.D., J.C. Chang, A.K. Pandey, C.M. Sassetti and D.R. Sherman, 2009. Role of cholesterol in *Mycobacterium tuberculosis* infection. Ind. J. Exp. Biol., 47: 407-411.

Parasa, V.R., M.J. Rahman, A.T. Ngyuen Hoang, M. Svensson and S. Brighenti *et al*., 2013. Modeling *Mycobacterium tuberculosis* early granuloma formation in experimental human lung tissue. Dis. Model Mech. DOI: 10.1242/dmm.013854

Pandey, A.K. and C.M. Sassetti, 2008. Mycobacterial persistence requires the utilization of host cholesterol. Proc. Natl. Acad. Sci. USA, 105: 4376-4380. DOI: 10.1073/pnas.0711159105

Pelosi, A., D. Smith, R. Brammananth, A. Topolska and H. Billman-Jacobe *et al*., 2012. Identification of a novel gene product that promotes survival of *Mycobacterium smegmatis* in macrophages. PLoS One 7: e31788-e31788. Doi: 10.1371/journal.pone.0031788

Rajni and L.S. Meena, 2011. Unique characteristic features of *mycobacterium tuberculosis* in relation to immune system. Am. J. Immunol., 7: 1-8. DOI: 10.3844/ajisp.2011.1.8

Rathor, N., A. Chandolia, N.K. Saini, R. Sinha and R. Pathak *et al*., 2013. An insight into the regulation of *mce4* operon of *mycobacterium tuberculosis*. Tuberculosis, 93: 389-397. Doi: 10.1016/j.tube.2013.03.007

Rhee, W.J., P.J. Santangelo, H. Jo and G. Bao, 2008. Target accessibility and signal specificity in live-cell detection of BMP-4 mRNA using molecular beacons. Nucl. Acid. Res., 36: e30-e30. DOI: 10.1093/nar/gkn039

Saini, N.K., M. Sharma, A. Chandolia, R. Pasricha and V. Brahmachari *et al*., 2008. Characterization of *Mce4A* protein of *Mycobacterium tuberculosis*: Role in invasion and survival. BMC Microbiol., 8: 200-200. DOI: 10.1186/1471-2180-8-200

Santangelo, P., N. Nitin, L. LaConte, A. Woolums and G. Bao, 2006. Live-cell characterization and analysis of a clinical isolate of bovine respiratory syncytial virus, using molecular beacons. J. Virol., 80: 682-688. DOI: 10.1128/JVI.80.2.682-688.2006

Shea, K.M., J.S. Kammerer, C.A. Winston, T.R. Navin and C.R. Horsburgh, Jr., 2014. Estimated rate of reactivation of latent tuberculosis infection in the United States, overall and by population subgroup. Am. J. Epidemiol., 179: 216-225. DOI: 10.1093/AJE/KWT246

Senaratne, R.H., B. Sidders, P. Sequeira, G. Saunders and K. Dunphy *et al*., 2008. *Mycobacterium tuberculosis* strains disrupted in mce3 and *mce4* operons are attenuated in mice. J. Med. Microbiol., 57: 164-170. DOI: 10.1099/jmm.0.47454-0

Ulrichs, T., G.A. Kosmiadi, S. Jorg, L. Pradl and M. Titukhina *et al*., 2005. Differential organization of the local immune response in patients with active cavitary tuberculosis or with nonprogressive tuberculoma. J. Infect. Dis., 192: 89-97. DOI: 10.1086/430621

Unlap, M.T. and R.S. Jope, 1997. Dexamethasone attenuates NF-kappa B DNA binding activity without inducing I kappa B levels in rat brain in vivo. Brain Res. Mol. Brain. Res., 45: 83-89. 10.1016/S0169-328X(96)00240-9

Van der Geize, R., K. Yam, T. Heuser, M.H. Wilbrink and H. Hara *et al*., 2007. A gene cluster encoding cholesterol catabolism in a soil actinomycete provides insight into *mycobacterium tuberculosis* survival in macrophages. Proc. Nat. Acad. Sci. USA, 104: 1947-1952. DOI: 10.1073/pnas.0605728104

Van Der Oost, J. and S.J. Brouns, 2009. RNAi: Prokaryotes get in on the act. Cell, 139: 863-865. DOI: 10.1016/j.cell.2009.11.018

Walter, N.D., J. Painter, M. Parker, P. Lowenthal and J. Flood *et al*., 2014. Persistent latent tuberculosis reactivation risk in United States immigrants. Am. J. Respir Crit. Care Med., 189: 88-95. DOI: 10.1164/rccm.201308-1480OC

WHO, 2013. Globel tuberculosis report 2013. WHO,

Wiedenheft, B., S.H. Sternberg and J.A. Doudna, 2012. RNA-guided genetic silencing systems in bacteria and archaea. Nature, 482: 331-338. DOI: 10.1038/nature10886

Xu, G., Y. Li, J. Yang, X. Zhou and X. Yin *et al*., 2007. Effect of recombinant *Mce4A* protein of mycobacterium bovis on expression of TNF-alpha, iNOS, IL-6 and IL-12 in bovine alveolar macrophages. Mol. Cell Biochem., 302: 1-7. DOI: 10.1007/s11010-006-9395-0

Xue, Y., R. An, D. Zhang, J. Zhao and X. Wang *et al*., 2011. Detection of survivin expression in cervical cancer cells using molecular beacon imaging: New strategy for the diagnosis of cervical cancer. Eur. J. Obstet Gynecol. Reprod. Biol., 159: 204-208. DOI: 10.1016/j.ejogrb.2011.06.038

Zhang, J.M., J. Guo, X. Tu, Z.H. Shi and J.J. Hao *et al*., 2007. Protective effect of Huaxia shallot preparation on human umbilical vein endothelial cell injury induced by oxidized low density lipoprotein and its mechanism. Zhong Xi Yi Jie He Xue Bao, 5: 675-680.

Zhou, C., Y. Mao, Y. Sugimoto, Y. Zhang and N. Kanthamneni *et al*., 2011. SPANosomes as delivery vehicles for small interfering RNA (siRNA). Mol. Pharm. DOI: 10.1021/mp200426h