USING *mce4* siRNA MOLECULAR BEACONS FOR DETECTION AND ERADICATION OF MYCOBACTERIAL INFECTION IN MACROPHAGES

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USING mce4 siRNA MOLECULAR BEACONS FOR DETECTION AND ERADICATION OF MYCOBACTERIAL INFECTION IN MACROPHAGES

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BIOCHEMISTRY AND MOLECULAR GENETICS

ABSTRACT

Tuberculosis (TB) continues to pose a significant threat to today’s society and is mediated by the pathogen Mycobacterium tuberculosis (Mtbb). The ability of Mtbb to invade and survive within macrophages of the pulmonary granuloma is attributed to the product of the mammalian cell entry (mce) genes whose operon, mce4, encodes a cholesterol transporter that helps transport host lipids into the bacterium that allows the bacterium to survive for years during chronic infection. Currently, there are no rapid and reliable tests for the detection and complete eradication of latent TB. Therefore, we propose and tested the hypothesis that mycobacterial infection in macrophages can be detected and eradicated using siRNA molecular beacons against mce4 operon mRNA. This hypothesis was tested in U937 cells infected or not infected with E. coli stably expressing individual mce4 genes from Mycobacterium smegmatis (E. coli-Ms4) and with Mycobacterium smegmatis (Ms) in the following specific aims: 1) to demonstrate that mce4 genes confer varying degrees of virulence, 2) to demonstrate that a mce4 siRNA molecular beacon can localize mycobacterial infection in macrophages, 3) to demonstrate that a mce4 siRNA molecular beacon can inhibit the mce4 mRNA transcript, and 4) to demonstrate that a mce4 siRNA molecular beacon can attenuate mycobacterial infection in macrophages. Our results with E. coli expressing individual M. smegmatis or Mtbb
mce4 operon genes showed that mce4A gene conferred the greatest degree of virulence to its host in comparison to other mce4 genes in the operon. We were then able to design a siRNA molecular beacon against the mce4A mRNA of M. smegmatis which was then successfully used to localize mycobacterial infection in macrophages. Using a GFP expressing lentiviral vector we were able to successfully transduce and stably express the mce4A siRNA molecular beacon construct in macrophages infected with either E. Coli expressing mce4A gene (E. Coli-4A) or M. smegmatis. Our invasion assay with macrophages stably expressing mce4A siRNA showed that the siRNA treatment attenuated E.coli-4A infection in macrophages at 3, 6, 24, and 48 hours 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 hours by 94.8%, 70.3%, 98.9%, and 93.4%, respectively. The degree of attenuation of E. coli-4A mce4A mRNA levels was compared between 3, 6, and 24hrs against that at 0hr and the results were found to be 0%, 81%, 40%, and 36%, respectively. The findings of these studies show proof of concept that latent TB infection can be rapidly and accurately detected and eradicated in vitro.
DEDICATION

I dedicate this work in memory of my late father, Njaralacattu Scaria George, and my mother, Chechamma George, both of who inspired me to pursue the path of knowledge from a young age. This work is also dedicated to my beloved wife, Elsa George, and my wonderful kids Leah, Leon, and Laura George, for whom I strive to attain the very best in me.
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LIST OF ABBREVIATIONS

ABC       ATP-binding cassette
AIDS      Acquired immune deficiency syndrome
ANOVA     Analysis of variance
ATCC      American Type Culture Collection
ATP       Adenosine triphosphate
BMP-4     Bone morphogenetic protein 4
Bp        Base pair
CD        Cluster of differentiation
CDC       Centers for Disease Control
cDNA      Complementary deoxyribonucleic acid
CFU       Colony forming units
CMV       Cytomegalovirus
DMEM      Dulbecco's modification of eagle's medium
E. coli   Escherichia coli
E. coli-4A Escherichia coli clone expressing MS mce4A gene
ECL       Enhanced chemiluminescence
EDTA      Ethylenediaminetetraacetic acid
EDTA      Isopropyl-β-D-thio-galactoside
EGFP      Enhanced green fluorescent protein
ER        Endoplasmic reticulum
ESAT      Early secreted antigen
FBS       Fetal bovine serum
FRET      Fluorescence resonance energy transfer
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric acid</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>IDT</td>
<td>Integrated DNA Technologies</td>
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<td>IFN-γ</td>
<td>Interferon gamma</td>
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<td>IGRA</td>
<td>Interferon gamma release assay</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
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<tr>
<td>LAM</td>
<td>Lipoarabinomannan</td>
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<td>LB</td>
<td>Luria-Bertani broth</td>
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<td>LTB</td>
<td>Latent tuberculosis</td>
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<tr>
<td>MB</td>
<td>Molecular beacon</td>
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<td>MCE</td>
<td>Mammalian cell entry</td>
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<td>MOI</td>
<td>Multiplicity of infection</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>MS</td>
<td>Mycobacterium smegmatis</td>
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<td>MTB</td>
<td>Mycobacterium tuberculosis</td>
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<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>OADC</td>
<td>Oleic acid, albumin, glucose, catalase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PDIM</td>
<td>Phthiocerol dimycocerosate</td>
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<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>pH</td>
<td>Potential of hydrogen</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<td>PknG</td>
<td>Protein kinase G</td>
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<td>PMA</td>
<td>Phorbol myristate acetate</td>
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<tr>
<td>PMO</td>
<td>Phosphorodiamidate morpholino oligo</td>
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<td>PNA</td>
<td>Peptide nucleic acid</td>
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<td>PPD</td>
<td>Purified protein derivative</td>
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<td>PS-ODN</td>
<td>Phosphorothioates oligodeoxynucleotides</td>
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<td>PVDF</td>
<td>Polyvinylidifluoride</td>
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<td>RNase</td>
<td>Ribonuclease</td>
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<td>RNS</td>
<td>Reactive nitrogen species</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RPMI</td>
<td>Roswell park memorial institute medium</td>
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<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
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<td>SapM</td>
<td>Secreted acid phosphatase</td>
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<td>SBP</td>
<td>Surface binding protein</td>
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<td>SDW</td>
<td>Sterile deionized water</td>
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<td>siRNA</td>
<td>Short interfering ribonucleic acid</td>
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<tr>
<td>TACO</td>
<td>Tryptophan aspartate-containing coat protein</td>
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<td>TGN</td>
<td>Trans golgi network</td>
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<tr>
<td>TIRFM</td>
<td>Total internal reflection fluorescence microscopy</td>
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<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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<td>US</td>
<td>United States</td>
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<td>V-ATPase</td>
<td>Vesicular adenosine triphosphatase</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<td>XDR-TB</td>
<td>Extensively drug-resistant tuberculosis</td>
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INTRODUCTION

Statement of Purpose

The aim of this research was to test the hypothesis that mycobacterial infection in macrophages can be detected and eradicated using siRNA molecular beacons against mce4 operon mRNA. This hypothesis was tested in U937 cells infected or not infected with E. coli stably expressing individual mce4 genes from Mycobacterium smegmatis (E. coli-Ms4) and with Mycobacterium smegmatis (Ms) in the following specific aims: 1) to demonstrate that mce4 genes confer varying degrees of virulence, 2) to demonstrate that a mce4 siRNA molecular beacon against this gene can localize mycobacterial infection in macrophages, 3) to demonstrate that this mce4 siRNA molecular beacon can inhibit the mce4 mRNA transcript, and 4) to demonstrate that this mce4 siRNA molecular beacon can attenuate mycobacterial infection in macrophages. This dissertation is divided into a number of sections and each section consists of a manuscript that has either been published or is to be submitted shortly. The introduction consists of a review paper that will be submitted within the next month. The first chapter consists of a manuscript that has been published in the Journal of Biotechnology and Biomaterials. The second chapter consists of a manuscript that was published in the American Journal of Biotechnology and Biochemistry and the third chapter consists of a manuscript that will be submitted shortly.
THE ROLE OF siRNA MOLECULAR BEACONS IN DETECTING AND ATTENUATING MYCOBACTERIAL INFECTION IN MACROPHAGES

by

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Manuscript for journal submission
Format adapted for dissertation
Abstract

Tuberculosis is one of the leading infectious diseases plaguing mankind and is mediated by the facultative pathogen, *Mycobacterium tuberculosis* (MTB). Once the pathogen enters the body, it subverts the host immune defenses and thrives for extended periods of time within the host macrophages in the lung granulomas, a condition called latent tuberculosis (LTB). Persons with LTB are prone to reactivation of the disease when the body’s immunity is compromised. Currently there are no reliable and effective diagnosis and treatment options for LTB, which necessitates new research in this area.

The mycobacterial proteins and genes mediating the adaptive responses inside the macrophage is largely yet to be determined. Over the recent years, it has been shown that the *mce* operon genes are important in the invasion of the mammalian host cell by the mycobacterium and for establishing a persistent infection in both *in vitro* and in mouse models of tuberculosis. The YRBE and MCE proteins encoded by the MTB *mce* operons have structural homology to the permeases and the surface binding protein (SBP) of the ABC transports respectively, and appear to be consistent with their cell surface location and proposed role in cell invasion at cholesterol rich regions and immunomodulation. The *mce4* operon is also thought to encode a cholesterol transport system that enables the mycobacterium to derive both energy and carbon from the host membrane lipids and possibly generating virulence mediating metabolites, thus enabling the bacteria in its long term survival within the granuloma. Various deletion mutation studies involving individual or whole *mce* operon genes have shown to be conferring varying degrees of attenuation of infectivity or at times hypervirulence to the host MTB, with the deletion of *mce4A* operon gene conferring the greatest degree of attenuation of virulence. Antisense
technology using synthetic siRNAs has been used in knocking down genes in bacteria and over the years this has evolved into a powerful tool for elucidating the roles of various genes mediating infectivity and survival in mycobacteria. Molecular beacons are a newer class of antisense RNA tagged with a fluorophore/quencher pair and their use for \textit{in vivo} detection and knockdown of mRNA is rapidly gaining popularity.
Epidemiology of Latent Tuberculosis

The year 2005 marked the 100th anniversary since Robert Koch got the Nobel prize for his work on tuberculosis (TB) and yet more than one hundred years later the World Health Organization (WHO) has reaffirmed its designation of tuberculosis as a global emergency (WHO 2010). Tuberculosis still remains a pandemic, infecting one-third of the world’s population and killing millions of people each year. Estimates are that a tuberculosis death occurs every minute. According to recent estimates of WHO, nearly 9 million people fell ill with TB in 2012, including 1.3 million TB-related deaths worldwide (Eurosurveillance editorial 2013). Incidentally, TB and reactivation of latent TB has turned out to be the leading cause of death for people who are infected with HIV. At the same time, according to the Centers for Disease Control (CDC), a total of 9,945 TB cases (a rate of 3.2 cases per 100,000 persons) were reported in the United States in 2012 (Centers for Disease and Prevention 2013). More than 80% of tuberculosis (TB) cases in the United States is from reactivation of latent TB infection (Horsburgh and Rubin 2011).

Tuberculosis is a disease that spreads from person to person through the air and is mediated by the pathogen *Mycobacterium tuberculosis*. The tuberculosis bacillus was discovered in 1882 and has been the subject of extensive research since then. There is still much to be learned about the nature of this organism, its virulence properties, and its response to host defenses. TB affects the lungs mainly, but can also have other target organs such as brain, spine and the kidneys. When a person with infectious disease coughs or sneezes, droplets containing *M. tuberculosis* are released into the air and when other person breaths in this infected droplet nuclei, he or she may
become infected. However, not everyone infected with TB bacteria becomes sick: there are two TB-related conditions that exist: latent TB (LTB) infection and active TB disease. People with latent TB infection do not feel sick and do not present with any symptoms. In LTB cases, even though they are infected with the mycobacterial pathogen, they do not have active TB disease. Overall, it has been shown that about 90% of the people infected with MTB will have LTB infection, and given the right conditions, 10% will eventually go on to have full-blown active TB at a later stage in their life (Kumar and Robbins 2007). About half of those people who develop TB will do so within the first two years of infection. For persons whose immune systems are weak, especially those with HIV infection, the risk of developing TB disease from LTB activation is considerably higher than for persons with normal immune systems. Also of special concern are persons infected by someone with extensively drug-resistant TB (XDR TB) and who later develop TB disease (Centers for Disease and Prevention 2013).

Thus, this ancient human adversary continues to be a challenge in all aspects of medical care, from prevention to diagnosis and therapy.

Need for Improved Diagnostic and Therapeutic Methods for Latent Tuberculosis

Currently, there are no tests available to directly detect in vivo the presence of latent MTB in an affected individual and assessment of latent infection involves an imperfect approach of measuring the host immune response to mycobacterial infection (Horsburgh and Rubin 2011). On the contrary, active tuberculosis infection is diagnosed by finding Mycobacterium tuberculosis bacteria in a clinical specimen taken from the patient. A definitive diagnosis of TB can be made only by culturing MTB from the
specimen, even though the results from this may take four to eight weeks for a conclusive answer. Other diagnostic methods for TB include chest x-rays, patient sputum smear microscopy, Polymerase Chain Reaction (PCR) testing, immunological memory-based tests including the less specific purified protein derivative (PPD/tuberculin) skin test and more specific IFN-γ release assays (IGRA), phage amplification assays, solid and automated liquid cultures, as well as several tests for antibiotic resistance. These tests can only strongly suggest the presence of active tuberculosis or latent tuberculosis as a diagnosis but they cannot confirm the presence of the bacteria in the body. Although dependable and reasonably fast in high-income countries, reliable and rapid diagnosis of latent TB is a major challenge in resource-poor settings, and even in parts of developed countries, especially in areas where immunodeficiency diseases like AIDS are more endemic. In many cases, patients have to undergo time-consuming multiple testing before reaching an apparent diagnosis. This testing deficiency can be especially critical when trying to identify high-risk individuals for prophylactic regimen, and also for identifying and managing extrapulmonary TB sites in HIV co-infected patients (Chakraborty and Chakraborty 2000).

Active TB cases are treated with a combination of four first-line antibiotics for two months, followed by two drugs for four months (Caminero, Sotgiu et al. 2010). These first line drugs include rifampicin, isoniazid, pyrazinamide, and ethambutol. These drugs are effective mainly in actively dividing bacilli and its effectiveness in treating latent tuberculosis, where the bacilli are dormant, has not yet been proven. The treatment of latent tuberculosis is usually long term with the intent of sterilizing the non-replicative or slowly replicating bacteria (Ehlers 2009). If the particular mycobacterial strain is
resistant to the first line drugs, then treatment is escalated for up to 18 months with five lines of available drugs. Surgery is also performed as a last resort if treatment fails due to drug resistance. Since the chemotherapy regimen for active and latent TB infections usually spans many months, poor patient compliance rates is a major issue contributing to the emergence of resistant strains (Horsburgh and Rubin 2011). There is an urgent need for faster and less expensive tests to confirm latent TB cases in order to manage this global epidemic.

Developing a direct MTB imaging screening tool for the asymptomatic population along with novel treatment strategies is vital to our fight against tuberculosis. This is especially true for high-risk categories with LTB such as people who inject drugs using unsanitary needles, residents and employees of high-risk congregate settings, medically under-served and low-income populations, high-risk racial or ethnic minority populations, children exposed to adults in high-risk categories, patients immunocompromised by conditions such as HIV/AIDS, people who take immunosuppressant drugs, and health care workers serving these high-risk clients (Griffith and Kerr 1996). Developing a direct MTB imaging screening tool with combined therapeutic applications for the asymptomatic population is going to have vitally important and far reaching impact in the fight against tuberculosis.

**Spectrum of Host Immune Response against MTB and Development of Latent Tuberculosis**

Humans are the only natural host of MTB and are highly susceptible to MTB infections. Even low load (5-10 bacilli) is capable of mediating a primary infection (Smith, Wiegeshaus et al. 1966). The initial interaction of the MTB with the host
involves alveolar macrophages, which is the only known cell type to harbor MTB in vivo (Filley and Rook 1991). The interaction of the host immune response with MTB can be divided into 4 general types of events (Schlesinger and DesJardin 2004): 1. Primary infection event involving the invading MTB, 2. Events that would promote the dissemination and progressions of the MTB infection, 3. Development of an adaptive immunity that would lead to the containment MTB infection, and 4. Interplay of protective immunity involved in latency vs. immunologic compromises leading to reactivation of MTB infection (Fig. 1).

Phagocytosis of the MTB by alveolar macrophages followed by its intracellular growth initiates the cascade of immune events of the primary infection (Hossain and Norazmi 2013). Activation of the components of the innate immunity, the recruitment of various classes of monocytes and lymphocytes to the site of infection, and the final development of specific immunity allow for the containment of infection (Fig. 2).

The hallmark of latent TB is the granulomatous lung parenchymal lesions and their draining lymph nodes which is called the “Ghon complex”. The events leading to the formation of granuloma begins when the MTB is inhaled into the lungs, and the bacterium is ingested by phagocytosis by resident alveolar macrophages and tissue dendritic cells. The infected cells release proinflammatory cytokines that help recruit more immune cells to the site of infection. The cytokines IL-12 and IL-18 from the infected cells induce NK cell activity, which in turn produce IFN-γ that helps active macrophages to produce TNF-α. Through the actions of these cytokines and chemokines, other immune cells are recruited leading to the formation of the granulomas (North and Jung 2004, Korbel, Schneider et al. 2008).
Fig. 1. Spectrum of host immune responses against MTB
Fig.2. Latent TB pathogenesis and transmission profile. Following MTB exposure, alveolar macrophages of the innate immunity phagocytize the inhaled bacteria. In immunocompromised individuals, MTB exposure leads to active TB. This leads to a pro-inflammatory response and recruitment of cells of the adaptive immunity, and the formation of a granuloma, where the bacteria can be contained for long periods of time. Inadequate adaptive response at any time will progress to the reactivation and development of active TB.
In the granuloma, the macrophages further differentiate into epitheloid cells and foamy macrophages and are surrounded by lymphocytes and an outer layer of fibroblasts and matrix proteins. The morphology of the lung granuloma is characterized by a central necrotic core surrounded by concentric layers of macrophages, epitheloid cells, multinucleated Langhans giant cells, and lymphocytes (Boros 1978, Mariano 1995). Containment of MTB at the site of primary infection by a cellular wall and a fibrotic outer layer prevents the pathogen from dissemination throughout the host and focuses the immune response to the site of mycobacterial persistence (Fig 3). Successful containment of the pathogen to the site of the primary lesion results in latent infection, which morphologically appears in chest x-rays as calcified granulomatous lesions (Ulrichs and Kaufmann 2006).

The precise site in which dormant MTB organisms persist during latent TB remains to be solved (Ulrichs and Kaufmann 2006). Studies over the last few decades suggest that the pathogen is present in macroscopically normal tissue outside the necrotic centers of granulomas (Feldman and Baggenstoss 1939). The preferred localization of dormant MTB during latency is the peripheral rim of the leukocyte infiltrate in the tuberculous lesion (Ulrichs, Lefmann et al. 2005).

During latency, a dynamic balance between the host immune response and the MTB pathogen is maintained. The granuloma is surrounded by lymphocyte infiltration, where the direct cross-talk between MTB (latent or active) and the host immune response takes place (Fenhalls, Wong et al. 2000, Ulrichs, Kosmiadi et al. 2004). The human tuberculous granulomas thus have an area of central necrosis, which provides the nutritional source for persisting mycobacteria, surrounded by a dense leukocyte wall.
**Fig 3. TB Granuloma.** A granuloma sequesters MTB infected macrophages and is surrounded by immune cells, predominantly CD4+ helper T lymphocytes. Some infected macrophages fuse to form foamy giant cells. The infected macrophages and giant cells present antigens to T cells and activate them to produce a variety of cytokines and chemokynes, and also kill the infected macrophage and the MTB. The chemokines also serve to recruit additional T cells to the granuloma from the circulating blood. IFN-γ activates the macrophages to kill the intracellular MTB by generating reactive oxygen species (ROS) and reactive nitrogen species (RNS) intracellularly. The center of the granuloma is filled with cell debris and both live and dead MTB spilled from dead macrophages (caseation), all of which form a central hypoxic necrotic core. A sheath of collagen fibers produced from lung fibroblasts surround the granuloma.
preventing mycobacterial spread. In latent TB, the persisting non-progressive tuberculous lesions (tuberculomas) are surrounded by highly vascularized tissue (Ulrichs, Kosmiadi et al. 2005) which enables the targeting of latent MTB with systemically delivered drugs.

**Post-Phagocytic Molecular Events following Mycobacterial Entry**

Once entering the host, the ability of MTB to survive decades within the body of the host by subverting the host immune defenses is of continued intrigue and fascination. The precise mechanism of how the bacteria is able to achieve this long term dormancy leading to latent tuberculosis is still unknown, however, recent advances in mycobacterial molecular biology revealed some of the bases of these processes.

Macrophages are the major components of the innate host defense, and they do this by pathogen recognition, ingestion and killing of foreign microbes that enter the body including pathogenic and non-pathogenic mycobacteria. The pathogenic mycobacteria have developed numerous strategies to subvert the host immune defenses and evade the destructive action of the macrophages, eventually surviving within this normally inhospitable cell for long periods of time thus resulting in the disease (Koul, Herget et al. 2004). The surviving bacteria within the macrophages can be in a latent state with stationary growth or, given the right conditions in an immunocompromised host, can switch to a metabolically active state that facilitates proliferation, dissemination and active disease.

Upon gaining entry into the body, most non-pathogenic microbes get phagocytosed by the macrophage into a phagosome. Within the phagosome the invading microbe gets exposed to high levels of reactive oxygen species (ROS) and reactive
nitrogen species (RNS). The phagosome then goes on to mature and fuse with the organelles of the endocytic pathway, thereby acquiring surface molecular markers which leads to the acidification of the phagosome to pH 5 as well as gaining hydrolytic enzymes that digest the invading microbe (Denis 1991, Chan, Xing et al. 1992) (Fig 4a). MTB, however, has developed several ways to manipulate the macrophage to avoid killing and creates a favorable environment for replication (Fig 4b). This is mainly by inhibiting several aspects of phagosomal maturation, including fusion and fission events along the endocytic pathway and the recruitment of vacuolar H⁺-ATPases (Sturgill-Koszycki, Schlesinger et al. 1994, Kuehnel, Goethe et al. 2001). The MTB carrying phagosome retains characteristics of an early phagosome with regard to its pH (about 6 – 6.5), presence of Rab5 (a Rho-GTPase directing endosomal trafficking and mediating fusion between phagosomes and other organelles), and continued access to other recycling endosomes (Via, Deretic et al. 1997, Rohde, Yates et al. 2007), but it lacks mature hydrolases (Vergne, Chua et al. 2004) and cathepsins, with interactions between the phagosome and the trans Golgi Network (TGN) (carrying cargo such as immature cathepsins) blocked (Vergne, Chua et al. 2003). Several MTB products are thought to modulate the prevention of phagosomal maturation, including the mycobacterial cell envelope components such as lipoarabinomannan (LAM), trehalose dimycolate and sulpholipids, as well as the phosphatase SapM and the kinase PknG (Rohde, Yates et al. 2007), and the secreted protein ESAT-6 (Tan, Lee et al. 2006). The exact mechanism behind the inhibition of phagosomal maturation by the mycobacteria is yet to be completely elucidated.
Studies have shown that cholesterol is a necessary component for the uptake of the MTB into the macrophage and for the inhibition of phagosomal maturation (Gatfield and Pieters 2000) (Fig. 4b). A host protein associated with the cell membrane called tryptophan-aspartate containing coat protein (TACO) is recruited and retained in the phagosomes harboring mycobacteria, thereby preventing the bacterial delivery to lysosomes (Ferrari, Langen et al. 1999). TACO is an actin-binding protein seen associated with cholesterol rich regions of the host macrophage plasma membrane (Gatfield and Pieters 2000). The mycobacterium within the phagosome is somehow able to prevent the removal of the TACO coat protein which prevents the fusion of phagosome with lysosome (Ferrari, Langen et al. 1999). Moreover, it has been shown that TACO-mediated uptake of mycobacteria depends on cholesterol (Gatfield and Pieters 2000, Anand and Kaul 2003, Kaul, Anand et al. 2004). The mycobacterial proteins and genes mediating these adaptive responses inside the macrophage is largely yet to be determined. *M. tuberculosis*’ unique ability to utilize cholesterol, a component of cell membranes, also plays a role in its persistence (Pandey and Sassetti 2008). In the nutrient-deficient intracellular environment, MTB adapts its metabolism, shifting from carbohydrate-based to fatty acid-based (Munoz-Elias and McKinney 2005). Studies have shown that MTB utilize cholesterol for its energy needs as well as for the biosynthesis of virulence-associated lipid PDIM (Pandey and Sassetti 2008). In addition, increasing number of reports indicate that MTB metabolizes cholesterol during host infections and that degradation of this sterol leads to products that contribute to long-term survival of MTB in the host (Pandey and Sassetti 2008, Miner, Chang et al. 2009, Forrellad, Klepp et al. 2013). Furthermore, because the cholesterol catabolism pathway requires a large
Fig. 4a. Stages of phagosomal maturation following non-pathogenic bacteria uptake. Upon entering into the body, most non-pathogenic microbes are phagocytized by the macrophage into a phagosome which then goes on to mature by fusing with the vesicles of the endocytic pathway and to finally fuse with lysosomes. These phagosomes undergo acidification due to the presence of proton-ATPase molecules from vacuolar membranes and the lysosomes, and this increased level of acidification activates the lysosomally derived acid hydrolases, cathepsins and other enzymes, along with reactive oxygen and nitrogen intermediates, to destroy the pathogen. Phagocytosis also initially triggers the recruitment of TACO around the particle to be ingested, as a result of the latter’s initial association with cell cortex microtubules, but is released prior to the lysosomal delivery of the bacteria.
Fig.4b. Effect of *M. tuberculosis* on phagosome maturation. Cholesterol serves as a docking site for the mycobacteria and its cell surface receptor there by facilitating its phagocytosis at cholesterol-rich regions. Cholesterol plays a crucial role in not only the entry of mycobacteria into macrophages but also mediates the phagososomal association of TACO (Coronin 1), a coat protein associated with cholesterol-rich regions which is actively retained on the phagosomal membrane housing the mycobacteria through a yet unknown mechanism, which prevents the degradation of the mycobacteria in the lysosomes.
number of oxygenases, it should be no surprise that MTB infects the lungs where oxygen concentration is the highest (Van der Geize, Yam et al. 2007).

**Mce Operons and their role in Mycobacterial Infection and Persistence**

Over the past few years it was shown that a DNA fragment from MTB cloned into *Escherichia coli* (*E. coli*) could mediate the bacteria’s entry and survival in mammalian cells (Arruda et al. 1993) and was named as the mammalian cell entry (*mce*) operon. The *mce* operon genes have been shown to be important in the invasion of the mammalian host cell by the mycobacterium and for establishing a persistent infection both *in vitro* and in mouse models (Flesselles et al. 1999, Senaratnes et al. 2008). The analysis of the complete genome sequence of *M. tuberculosis* in 1998 (Cole et al. 1998) showed that the *mce* operon is composed of a group of four homologous *mce* operons (*mce1, mce2, mce3*, and *mce4*). It was found that all the constituent *mce* genes in the four operons were arranged in an identical manner. Each of the operon contained eight genes, two genes preceding the *mce* genes are named *yrbEA* and *yrbEB* which encoded for integral membrane proteins and the six *mce* genes potentially encoding exported proteins (secreted or surface-exposed) thought to be important for the entry and survival of the pathogen in the mammalian cells (Cole et al. 1998). The four *mce* operons are widely seen throughout the genus Mycobacterium (Gatfield and Pieters 2000) and the general organization of the genes in each of the four operons are shown in Fig. 5.

The YRBE and MCE proteins encoded by the MTB *mce* operons have structural homology to the permeases and the surface binding protein (SBP) components of the ABC transporters, respectively (Casali and Riley 2007). The typical ABC permease
Fig. 5. The organization of the mce operons. The operon structures of the four mce operons with their constituent genes are shown. The grey arrows represent yrbE genes and the blue arrows represent the mce genes.
contains six trans-membrane helices with the C-terminus located on the cytoplasmic side of the membrane (Fig. 6a). The YRBE permease contains five or six transmembrane segments outside the C-terminus and the orientation of the N-terminal transmembrane helix may be either cytoplasmic or extracellular (Fig. 6b), suggesting a transmembrane transport role (Liu, Liu et al. 1999, Casali and Riley 2007).

The MCE protein, including MCE1A, 3A and 4A, but not MCE2A, make up a patch of 275-564 amino acid residues, with the hydrophobic stretch at the N-terminal anchored in the membrane, after folding and modification (Casali and Riley 2007), along with a 22 amino acid “invasion domain” near the C-terminal that is exposed to the outside the membrane (Das, Mitra et al. 2003, Mitra, Saha et al. 2005) (Fig. 6b). These characteristics are consistent with their cell surface location and proposed role in cell invasion and immunomodulation (Harboe, Christensen et al. 2002), however, the mechanism of interaction between YRBE and MCE proteins is not yet clear (Zhang and Xie 2011).

Phylogenetic studies have shown that the MCE proteins share between 30-70% amino acid identity to their inter-operon counterparts but only 16-26% identity with other MCE proteins encoded by the same operon. For example, there is 61% identity between MCE1A and MCE2A, however, it falls to 25% identity between MCE1B and MCE1C (Ahmad, Akbar et al. 1999, Tekaia, Gordon et al. 1999). Because of the multiple mce operons in the genome, it is proposed that they may have redundant or time dependent activities. This possibility is supported by the temporal transcriptional expression differences during different stages of in vitro growth between mce1 in comparison to mce3 and mce4 (Kumar, Bose et al. 2003). Differences are also seen with in vivo growing
Fig 6a. **Topology of typical ABC transmembrane permease.** ATP Binding Cassette (ABC) transport systems in both prokaryotes and eukaryotes consists of four domains, two cytoplasmic ABC domains and two hydrophobic membrane spanning permease domains. Each of the membrane spanning domain typically contain 6 hydrophobic transmembrane segments anchored in the cell membrane with the C- and N-termini located towards the cytoplasmic side. ABC transporters in prokaryotes that function as importers also typically require additional extracytoplasmic helper proteins called ‘substrate binding proteins’ for function.
Fig 6b. Predicted consensus topology of YRBE permease and MCE invasion domain. Each of the mce operons contain two YRBE domains that have structural homology to the permease domain of the ABC transporters. Each YRBE permease contains five or six transmembrane segments with the C-terminal end located on the extracytoplasmic side and the N-terminal end being either cytoplasmic or extracellular. The MCE protein, MCE4A, consists of a patch of 400 amino acid residues, with a hydrophobic stretch anchored in the cell membrane and a 22-amino acid invasion domain exposed outside to the membrane, which is predicted to be structurally similar to the substrate binding domain of ABC importers.
bacilli in that even though \textit{mce1}, \textit{mce3}, and \textit{mce4} transcripts are detectable up to 24 weeks post infection in rabbit lung tissue, only \textit{mce4} transcript is detectable 16 weeks post infection in guinea pig spleen (Kumar, Bose et al. 2003). The expression of \textit{mce2} was not detected under any conditions tested (Kumar, Bose et al. 2003).

Studies using \textit{mce} operon deletion or disruption mutants of MTB have demonstrated varying effects with the different \textit{mce} operons. Studies have shown that disrupting \textit{mce} operons lead to attenuation (Sassetti and Rubin 2003, Gioffre, Infante et al. 2005), while others have shown some degree of hypervirulence for the host MTB following the mutations (Shimono, Morici et al. 2003, Lima, Sidders et al. 2007). Deletion of \textit{mce} operons 3 and 4 attenuated MTB virulence in infected macrophages (Joshi, Pandey et al. 2006).

The \textit{mce4A} gene is the first among the six \textit{mce} genes in the \textit{mce4} operon that has been studied the most. It was shown that the MCE4A protein of \textit{mce4} operon is not only important for host cell invasion but also for survival of the MTB pathogen in human macrophages (Saini, Sharma et al. 2008, Chandolia, Rathor et al. 2014). Individual \textit{mce1}, \textit{mce2}, \textit{mce3}, and \textit{mce4} mutants administered intranasally or intravenously in mice have shown to result in lower bacterial burdens and slower mortality of the infected mice, with \textit{mce4} operon deletion showing the greatest effects on MTB virulence (Gioffre, Infante et al. 2005, Joshi, Pandey et al. 2006). The route of infection was also shown to be having an effect on the attenuation results in one study (Gioffre, Infante et al. 2005). Hypervirulence among \textit{mce1} mutants have been demonstrated in two separate studies when administered intranasally, intravenously, or intraperitoneally (Shimono, Morici et al. 2003, Lima, Sidders et al. 2007). The deletion mutants in all these studies, however,
were not identical in the nature of their deletions and had varied in their deletion sequences that possibly led to different polar effects on downstream genes, which may explain the discrepancy in the results of some of the deletion mutant studies.

Apart from its possible role in mediating host infection, it is proposed that at least some of the MCE4 proteins form an outer membrane channel that enables cholesterol to enter the cell, thereby enabling the mycobacterium to uptake host lipids vital for its survival during the prolonged latent infection (Niederweis, Danilchanka et al. 2010). Transposon Site Hybridization studies showed that certain MTB genes involved in the lipid metabolism were genetically linked to the \textit{mce4} operon genes (Joshi, Pandey et al. 2006). The \textit{mce4} has been shown to encode a cholesterol transport system that enables the mycobacterium to derive both carbon and energy from the host membrane lipids and also for possibly generating sterol metabolites mediating its long-term survival within the macrophage (Pandey and Sassetti 2008), with the \textit{mce4} expression progressively increasing as the latency phase advances (Rathor, Chandolia et al. 2013). Apart from the environmental stressors like hypoxia and surface stressors, cholesterol was also found to be an inducer of the \textit{mce4} operon (Rathor, Chandolia et al. 2013).

\textbf{Antisense Technology in Bacteria}

Hundreds of bacterial encoded short interfering RNAs (siRNA) have been reported over the past decade (Papenfort and Vogel 2009, Waters and Storz 2009, McClure, Tjaden et al. 2014). Majority of these siRNAs act by binding to their target mRNAs to bring about the repression. They broadly fall into two major categories: the ones that are encoded at locations that are farther away from the target gene (trans-acting)
and the ones that are transcribed from the DNA strand opposite another gene with perfect complementarity to the target gene (cis-acting). The trans-acting siRNAs generally share only limited complementarity with their target gene and are prone to have off-target effects. Trans-acting siRNAs are by far the most characterized bacterial siRNAs and have been shown to usually require the chaperon protein Hfq for base pairing (Aiba 2007). The cis-encoded siRNAs, or anti-sense RNAs, have perfect complementarity with their target gene and thus have more extensive and stronger base pairing. Among the reported bacterial antisense RNAs, some are short (siRNA), with around 100 nucleotides in length and are usually encoded by plasmids or bacteriophages, while some are chromosomally encoded and are longer, in some cases overlapping entire genes or corresponding to the 5’ or 3’ extension of the protein coding region of the mRNA. The 5’ untranslated region of the mogR mRNA in Listeria monocytogenes overlaps 3 genes on the opposite strand involved in the flagellar synthesis and serves as an example of chromosomally encoded long antisense RNAs (Toledo-Arana, Dussurget et al. 2009). The binding region of antisense RNA on the target mRNA can also vary and may overlap the 5’ end, or the 3’ end, or the middle, or even the entire gene coding region.

Antisense RNAs in the bacterial cell have been shown to repress many abnormal events in the cell such as transposons and toxic proteins. One of the first antisense RNA to be discovered in bacteria was the RNA-OUT of the transposon Tn10, which was shown to repress transposition by preventing the translation of transposase mRNA (Simons and Kleckner 1983). Antisense RNAs against transposase genes are also seen in Salmonella enterica (Padalon-Brauch, Hershberg et al. 2008, Sittka, Lucchini et al. 2008), Caulobacter crescentus (Landt, Abeliuk et al. 2008), and Listeria monocytogenes.
(Toledo-Arana, Dussurget et al. 2009). Thus an important function of bacterial antisense RNA, as in eukaryotes (Malone and Hannon 2009), appears to be the inhibition of transposition. An increasing number of antisense RNAs have also been shown to downregulate the expression of toxic proteins (Gerdes and Wagner 2007, Fozo, Makarova et al. 2010). Most of these repressed proteins are hydrophobic, small with less than 50 amino acids, and toxic at higher levels. An example of this tight repression of one such toxic protein is seen in *E. coli*, where the low levels of SymE protein is maintained by the LexA repressor of the SOS response, the SymR antisense RNA and the Lon protease (Kawano, Aravind et al. 2007). Some of the antisense RNAs to transposases and toxic genes, such as SymR, are expressed constitutively in the cell (Kawano, Aravind et al. 2007).

Antisense RNAs have also been shown to positively and negatively regulate the expression of various transcriptional regulators and other metabolic and virulence proteins in bacterial systems. For example, The 109 nucleotide *GadY* antisense RNA of *E. coli* overlaps the intergenic region of the dicistronic *gadXW* mRNA which encodes two transcription regulators of the acid stress response genes and the overexpression of the *GadY* RNA results in the processing of *gadXW* mRNA into two separate *gadX* and *gadW* transcripts leading to positive regulation (increased expression) of those genes (Opdyke, Kang et al. 2004, Tramonti, De Canio et al. 2008). On the other hand, in the nitrogen-fixing cyanobacterium *Anabaena sp. PCC 7120*, the ~2,200 nucleotide *alr1690-α-furA* antisense RNA spans the entire *alr1690* coding region and extends through the gene encoding the ferric uptake transcriptional regulator, FurA, into its promotor and regulator regions and it helps decrease *furA* expression and translation, thereby acting as
a negative regulator of iron absorption and nitrogen metabolism (Hernandez, Muro-Pastor et al. 2006). Similar regulatory RNAs controlling metabolic responses to environmental effects have been reported in many other bacterial systems (Rodionov, Vitreschak et al. 2004, Duhring, Axmann et al. 2006, Masse, Salvail et al. 2007, Recalcati, Minotti et al. 2010, Shioya, Michaux et al. 2011, Patenge, Billion et al. 2012, Jackson, Pan et al. 2013).

Another recently discovered phenomenon is the antisense-mediated gene regulatory switch in the bacteria called the “excludon”. This consists of a gene locus encoding an unusually long antisense RNA that spans divergent genes or operons with related or opposing functions. In such a regulatory system, the antisense RNA can inhibit the expression of one operon while functioning as an mRNA for the adjacent operon, thereby acting as fine-tuning regulatory switches in bacteria (Sesto, Wurtzel et al. 2013).

Antisense RNA has also been shown to regulate the expression of various structural and virulence factors in different bacteria. One example is the 1,200 nucleotide AmgR RNA encoded opposite the Salmonella enterica mgtCBR operon associated with its virulence and survival in macrophages (Lee and Groisman 2012). A number of other antisense RNAs modulating virulence and regulating host-pathogen interactions have been discovered in a variety of bacterial species over the years (Heroven, Bohme et al. 2008, Wadler and Vanderpool 2009, Caswell, Gaines et al. 2012, Koo and Lathem 2012, Postic, Dubail et al. 2012, Schmidtke, Findeiss et al. 2012, Shepherd, Li et al. 2013, Zeng, McNally et al. 2013). Antisense RNAs have also been found to impact other benign structural components including flagellar synthesis in Rhizobium (Torres-

The first complete experimental confirmation of short antisense RNAs in mycobacteria was published in 2009, which revealed 5 trans-acting and 4 cis-acting siRNAs in *M. tuberculosis* H37Rv in the context of pH and oxidative stress (Arnvig and Young 2009). By the end of 2013, a total of more than 200 endogenous antisense RNAs were experimentally identified in various mycobacteria, including 70 in *M. tuberculosis* (Warner, Savvi et al. 2007, DiChiara, Contreras-Martinez et al. 2010, Arnvig, Comas et al. 2011, Hartkoorn, Sala et al. 2012, McGuire, Weiner et al. 2012, Miotto, Forti et al. 2012, Pelly, Bishai et al. 2012, Houghton, Cortes et al. 2013, Tsai, Baranowski et al. 2013), 90 in *M. Bovis* (DiChiara, Contreras-Martinez et al. 2010, Golby, Nunez et al. 2013, Tsai, Baranowski et al. 2013), 9 in *M. avium* (Ignatov, Malakho et al. 2013), and 44 in *M. smegmatis* (DiChiara, Contreras-Martinez et al. 2010, Arnvig, Comas et al. 2011, Li, Ng et al. 2013, Tsai, Baranowski et al. 2013). From these recent studies, a stronger connection between mycobacterial pathogenesis and the levels of expression of the antisense RNAs have emerged but many new questions about their potential pathogenic versus housekeeping functions remain to be answered. The lack of identification of an Hfq homolog in mycobacteria prevents the current approach of coimmunoprecipitation, making the study of the role of antisense RNAs all the more difficult in this genus (Arnvig, Comas et al. 2011). Pandey et al. have proposed an alternative protein, Rv2367, as a potential RNA chaperon in place of Hfq (Pandey, Minesinger et al. 2011), however, studies are ongoing in this direction to find a functionally-equivalent chaperon or to get
around this issue (Li, Ng et al. 2013). Also, the role of mycobacterial antisense RNAs in regulating transposition is not as clear as in other bacterial systems like *E. coli*.

Antisense RNAs can act by repressing or modulating their target gene expression through many different ways, including transcription interference, transcription attenuation, degradation by endo- or exonucleases, or by blocking ribosome binding. When inducing transcription interference, the transcription of antisense RNA from one promoter hinders the RNA polymerase from either binding or extending the target gene transcript from the opposite strand (Callen, Shearwin et al. 2004, Andre, Even et al. 2008). This type of interference occurs only in Cis and does not involve base pairing. In transcription attenuation, the binding of the antisense RNA to the target RNA causes a conformational change creating a terminator structure in the mRNA leading to its premature termination of transcription (Stork, Di Lorenzo et al. 2007). Antisense RNAs can also affect the stability of the target mRNA by inducing or blocking its degradation. When employing endo- or exonucleases for gene regulation, the antisense RNA, upon binding to its target mRNA, induces or blocks a ribonuclease target site within the mRNA or can indirectly block the binding of the ribonuclease at a distant site. In many bacterial systems, two major endoribonucleases have been identified, the first being RNase III which cleaves double stranded RNA into two halves with different stabilities than the original transcript (Blomberg, Wagner et al. 1990, Gerdes, Nielsen et al. 1992, Vogel, Bartels et al. 2003, Carpousis, Luisi et al. 2009), and the second endoribonuclease reported being RNase E, which cleaves single stranded RNA. RNase E is a component of the multi-protein degradasome complex that can interact with Hfq and globally affect mRNA stability (Carpousis, Luisi et al. 2009, Saramago, Barria et al. 2014). It is not
precisely clear how the antisense RNA modulates RNase E activity, but the proposed mechanisms include the donation of its 5’ monophosphate to stimulate RNase E activity or physically block the RNase E recognition site by basepairing to downregulate activity (Celesnik, Deana et al. 2007). Other ribonucleases in bacteria have also been identified with more specialized functions, including RNase G (a non-essential paralog of RNase E) (Umitsuki, Wachi et al. 2001, Kaga, Umitsuki et al. 2002), RNase P (Alifano, Rivellini et al. 1994, Li and Altman 2003), RNase LS (Iwamoto, Lemire et al. 2008), RNase Z (Schilling, Ruggeberg et al. 2004, Perwez and Kushner 2006), RNase H (Anupama, Leela et al. 2011), RNase J1/J2 (Even, Pellegrini et al. 2005), and the recently characterized RNase Y (Shahbabilan, Jamalli et al. 2009). Many of these ribonucleases have already been characterized in various Mycobacteria, including *M. tuberculosis* and *M. smegmatis* (Akey and Berger 2005, Kovacs, Csanadi et al. 2005, Zeller, Csanadi et al. 2007, Csanadi, Faludi et al. 2009, Watkins and Baker 2010, Taverniti, Forti et al. 2011, Murdeshwar and Chatterji 2012, Abendroth, Ollodart et al. 2014, Herrmann, Stolt et al. 2014). Apart from these mechanisms, it has also been found that some antisense RNAs can physically block mRNA expression by binding to the Shine Dalgarno sequences of their target mRNA and prevent ribosome binding (Hernandez, Muro-Pastor et al. 2006, Kawano, Aravind et al. 2007, Papenfort and Vogel 2009), or they may indirectly modulate expression by altering the target mRNA conformation (Waters and Storz 2009). Finally, antisense RNAs can have dual functions by acting as both mRNAs and antisense RNAs or both cis- and trans- acting RNAs (Opdyke, Kang et al. 2004).

Two general mechanisms have been proposed for basepairing in antisense RNAs. The first type is a single-step mechanism in which the antisense RNA makes initial
contact with the target mRNA, which then leads to complete duplex formation (Kittle, Simons et al. 1989). The second type of mechanism is a multi-step system in which the initial transient interaction between the antisense RNA and mRNA is stabilized by a protein, followed by the formation of the more stable complete duplex (Tomizawa 1984, Tomizawa 1990). In many cases of base pairing for the antisense RNA, a stem-loop structure is found to be important, along with a ‘pyrimidine-uracil-any nucleotide-purine’ U-turn motif (Franch and Gerdes 2000).

Synthetic antisense RNAs are generally delivered either by expressing the antisense transcript from a gene introduced into the cell or by direct delivery of antisense oligonucleotides. Degradation of the antisense transcripts can be a problem for both these delivery approaches, however, this issue is mitigated by using sequences that form more stable hair-pin structures with paired ends (Nakashima, Tamura et al. 2006). In order to increase the stability and uptake, antisense RNAs have been modified in many ways, including the addition of peptide nucleic acid (PNA) or alternating 2’O-methyl to their backbones, switching ribose rings to morpholine rings (PMO), switching internucleoside bonds with phosphorothioates (PS-ODNs), or by conjugating cationic peptides to PNAs and PMOs (Good, Awasthi et al. 2001, Nikravesh, Dryselius et al. 2007, Rasmussen, Sperling-Petersen et al. 2007).

Antisense technology using synthetic siRNAs have been used as a powerful tool in knocking down genes in prokaryotes (and also in eukaryotes), including hepatitis G virus (Cao, Ren et al. 2005), influenza virus (Ge, McManus et al. 2003), picornavirus (Lim, Yuan et al. 2008), and Trypanosoma brucei (Zhang, Guo et al. 2007). When targeted to essential genes, they have been shown to inhibit growth of E. coli (Good,

Molecular beacons are a newer class of antisense RNA tagged with a fluorophore/quencher pair and their use for \textit{in vivo} detection and knockdown of mRNA is gaining popularity. Molecular beacon based short interfering RNA (MB siRNA) has recently been proven to be a powerful tool for therapeutic gene silencing because of its specificity, broad applicability, and high efficiency (Kim, Sohn et al. 2008, Hong, Zhang et al. 2010, Ilieva, Della Vedova et al. 2013). The on/off signals produced by the fluorophore/quencher pair depends on the conformational state of the MB (Fig 7). In the absence of the target mRNA, the stem brings the quencher in close proximity with the fluorophore and turns the fluorescence off with high quenching efficiency via
Fluorescence Resonance Energy Transfer (FRET). In FRET, the energy from the donor chromophore is transferred to an acceptor quencher near-by thus resulting in the absence of fluorescence. If the quencher and the fluorophore are far apart (following hybridization of the beacon to its target), then the quencher molecule will not be able to absorb the energy from the donor fluorophore and this would result in an increase in fluorescence. This technology has been used to detect mRNA expression in cells as well as for the detection and knockdown of telomerase expression in human breast cancer cells (Chang, Zhu et al. 2007), BMP4 mRNA in hedgehog signaling (Rhee, Santangelo et al. 2008), aromatase mRNA in breast cancer cells (Zhou, Mao et al. 2011), and for the detection and attenuation of MCE4A mediated \textit{M. smegmatis} infection in macrophages as discussed in section below (George, Unlap et al. 2012, George, Unlap et al. 2014).

\textit{M. smegmatis} as an in vitro Model for Studying Mycobacterial Infection and Persistence

The \textit{mce} operons are widely seen throughout the genus \textit{Mycobacterium} and a homolog of \textit{mce4} has been confirmed in the mycobacterial species \textit{M. smegmatis} (Haile, Caugant et al. 2002, Rathor, Chandolia et al. 2013). Even though \textit{M. smegmatis} is non-pathogenic, previous studies have shown that it can survive and multiply within macrophages in a pathogen-like manner by manipulating the host cell during initial stages by delaying phagosomal acidification and recruitment of V-ATPase (Kuehnel, Goethe et al. 2001, Anes, Peyron et al. 2006), thus making it a suitable model to study \textit{mce4 operon} mediated invasion and intracellular mycobacterial survival.
Fig. 7. Molecular beacon technology. Molecular beacons are hairpin shaped antisense RNAs that do not fluoresce in the absence of mRNA binding (left) while the fluorescence increases when the beacon hybridizes with its target mRNA (right).
The role of Molecular Beacons in Detecting and Attenuating Mycobacterial Infection in Macrophages

In order to determine the ability of a siRNA molecular beacon to detect and attenuate mycobacterial infection in macrophages, we first conducted experiments towards determining the most infective gene in the mce4 operon. Because of the slow growth rate of *M. tuberculosis* and also due to the high degree of homology between mce4 operons of mycobacteria (Haile, Caugant et al. 2002), the mce4 operon of the rapidly growing *M. smegmatis* was selected for our studies. Using gene specific primers with the reverse primer for each set excluding the termination codon, each of the mce4 genes, *mce4A, mce4B, mce4C, mce4D, and mce4F* were PCR amplified, cloned into the prokaryotic expression vector *pTrcHis2-TOPO* and stably expressed in *E. coli*. Western blot analyses with monoclonal antibodies against c-myc and 6xHis showed that the MCE4 proteins were expressed in host *E.coli*. We then conducted invasion assays in MCF7 breast cancer cells using *E. coli* clones expressing the *M. smegmatis* genes and the results showed that *mce4A-F* conferred virulence to its host *E.coli*. However, *mce4A* appeared to confer the earliest virulence to its host *E. coli* and the virulence was found to be sustained during the entire invasion period (72hr) (George, Unlap et al. 2012). These experiments were repeated using the mce4 operon genes of *M. tuberculosis*, by PCR amplifying each of the *mce4A-F* genes (Fig. 8), cloning into the prokaryotic expression vector *pTrcHis2-TOPO*, transformed into E. coli, and performing invasion assay using MCF7 breast cancer cells. Our results showed that, as with *M. smegmatis*, the *mce4A* gene conferred the greatest degree of virulence to its host *E. coli* (Fig. 9). Therefore, *mce4A* was selected as the target gene for designing a molecular beacon antisense RNA.
Fig. 8. PCR amplification of mce4A, mce4B, mce4C, mce4D and mce4F of *M. tuberculosis*. *Mce4* operon genes were PCR amplified from *M. tuberculosis* H37Rv using gene specific primers and resolved on 1% agarose gel. The forward primer spanned the first 21 nucleotides from the beginning of the open reading frame and the reverse primer covered 21 nucleotides spanning the complementary strand to the 3’ end of the gene. The termination codon was omitted so that the product, MCE4A-F, will be expressed with a 6XHis tag and a myc tag.
Fig. 9. Mtb-MCE4 proteins confer virulence to *E.coli*. *E.coli-Mtbmce4* clones were used to infect 2x10^6 MCF7 epithelial cells at an MOI of 10:1 for 2 hours. The level of infection was assessed by counting bacterial colony numbers at 24hrs, 48hrs and 72 hours post-infection (n=3).
An antisense RNA against the *mce4A* antisense molecular beacon RNA was designed to have a stem-loop structure, with the nucleotides in the stem complementary to each other to form a 5-base pair double stranded stem and the loop consisting of 20 nucleotides that are complementary to a region of the target *mce4A* mRNA in *M. smegmatis*. Also, conjugated to the 5’ and 3’ ends of this molecular beacon are the fluorophore TYE 665 and quencher Iowa Black RQ-SP, respectively. This molecular beacon design combined both detection and therapeutic capabilities as previous studies using beacons of such design have reported (Chang, Zhu et al. 2007, Rhee, Santangelo et al. 2008, Zhou, Mao et al. 2011). The rationale is 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 20 nucleotide loop will compete with the 5 nucleotide stem for hybridization to their target *mce4A* mRNA and the stem to its complementary pairs on the opposite ends of the target sequence. The hybridization potential of the loop to its target, based on the number of nucleotides within it (20 versus 5), will be greater than that of the strands for the stem. Hybridization of the loop to the *mce4A* mRNA will separate the fluorophore from the quencher thus inducing fluorescence (detection) and degradation (therapeutic) of the mRNA. Since the mycobacterium utilizes the product of *mce4A* for entry into macrophages and for its survival using host cholesterol for carbon and energy source transported through the MCE4 associated transporters (Xu, Li et al. 2007, Saini, Sharma et al. 2008, Senaratne, Sidders et al. 2008, Miner, Chang et al. 2009), the degradation of the *mce4A* mRNA will lead to its reduced survival. Our studies first tested the ability of the *mce4A* siRNA to detect its target *mce4A* mRNA in *M. smegmatis* and in macrophages infected with *M. smegmatis* and the results show that the molecular
beacon siRNA detected its target in *M. smegmatis* and in macrophages infected with *M. smegmatis*. Thus, we were able to show that a molecular beacon can be designed against one of the *mce4* operon genes in *M. smegmatis* that facilitates the detection of mycobacterial infection in macrophages.

Tests were carried out to test the ability of this siRNA molecular beacon to not only detect but also attenuate mycobacterial infection in macrophages. To this end, a GFP expressing lentiviral vector, *piLenti-siRNA-GFP*, was used to successfully transduce and stably express the *mce4A* siRNA molecular beacon construct in macrophages infected with either *E. Coli* expressing *mce4A* gene (*E. Coli-4A*) or *M. smegmatis*. Using confocal imaging and Western blot analyses with anti-GFP antibodies, our studies demonstrated stable expression of siRNA up to 48hrs post transduction and infection using the GFP reporter.

After confirming the expression of the GFP protein by fluorescence imaging and Western blot analyses, invasion assays were carried out to assess the effect of *mce4A* siRNA on mycobacterial infection in macrophages. Differentiated U937 macrophages were transduced with *piLenti-siRNA-GFP* phage for 24hrs followed by infection with *E.coli-4A* or *M. smegmatis* for 3 hours, and incubation for 0, 3, 6, 24, and 48hrs, respectively. The cells were extensively washed and lysed in 0.1% Triton-X 100 lysis buffer and the lysates were plated on either LB agar containing 100µg/ml ampicillin for *E. coli-4A* or 7H11 media for *M. smegmatis*. The degree of attenuation of *E. coli-4A* infection was compared between 3, 6, 24, and 48hrs against that at 0hr baseline 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 48hrs against that at 0hr
baseline and was found to be 94.8%, 70.3%, 98.9%, and 93.4%, respectively. Thus, our results showed that the mce4A siRNA was able to significantly attenuate both E. coli-4A and M. smegmatis infection in macrophages (George, Unlap et al. 2014).

Experiments were conducted to test the hypothesis that the mce4A siRNA molecular beacon can attenuate the mce4A mRNA levels in E. coli expressing mce4A gene within infected macrophages. RTPCR analysis was performed on lysates from differentiated U937 cells which were transduced with the piLenti-siRNA-GFP phage for 24hrs, followed by infection with E. coli-4A for 3hrs and incubation for 0, 3, 6, and 24hrs. The cells were washed, lysed and the intracellular bacteria were isolated and washed at each time point of incubation. The bacterial sample from each of the time points were lysed and the mRNA was isolated and purified using DNase 1 enzyme treatment. Reverse transcripts were generated using RTPCR and the cDNAs were amplified using gene specific primers for M. smegmatis mce4A and E. coli 16S rRNA gene as internal control. The degree of attenuation of mce4A mRNA levels was compared between 3, 6, and 24hrs against that at 0hr and the results were found to be 0%, 81%, 40%, and 36%, respectively using densitometry gel analysis. These results showed that mce4A siRNA was able to attenuate mce4A levels within infected macrophages as opposed to E. coli 16S rRNA internal positive control and the degree of attenuation of mce4A mRNA levels in E. coli-4A was found to be significant.

The studies conducted in our lab demonstrated that a molecular beacon can be designed against one of the mce4 operon genes in M. smegmatis that detect and attenuate mycobacterial infection in macrophages.
Antisense oligonucleotides, often mentioned as the pharmacology of the future (Nyce 2002), can bind to their mRNA targets with much greater specificity and avidity than can traditional drugs to their protein targets. Recent advances have enhanced their hybridization to target mRNA, reduced their overall toxicity with decreased susceptibility to cellular nucleases. The lung offers an exceptional target for direct antisense oligonucleotide delivery by inhalation, thereby achieving a bolus dose directly to the target site. Cationic lipids in the lung surfactants enhance the uptake of oligonucleotides into cells (Monkkonen and Urtti 1998). Penetration of the inhaled oligonucleotides into deeper tissues of the lung has been established by autoradiogram, surgical dissection & receptor quantification studies (Nyce and Metzger 1997). Further studies to test the hypothesis that mce4 siRNA respirable molecular beacons can localize and attenuate mycobacterial infection in pulmonary granulomas in animal models will take the fight against TB a long way in eradicating this versatile human pathogen.

**Conclusion**

The association of the mce operons, especially that of mce4, with mycobacterial invasion and latency is no longer considered casual and with strong evidences emerging over the recent years it can now be considered as a potent mediator of MTB infection and survival in its only human host. The mce invasion domain is equipped to mediate the entry and localization of the bacteria in the host macrophages at cholesterol rich regions creating cholesterol-associated protein coated phagosomes, thereby creating an ingenious mechanism for subverting the immune defenses. Another paradigm to the mycobacterial saga was added by the discovery that the MCE associated
protein, YRBE4 transporters, in conjunction with the MCE4 domains, transport cholesterol into the cell for its energy and carbon needs, which then possibly generates metabolites that can further mediate its latency in the host. Strategies like identifying the level of infectivity of individual mce operon genes and designing efficacious drugs like molecular beacon siRNAs against mce targets can aid in the simultaneous detection and eradication of this elusive human pathogen.
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A SHORT INTERFERING RNA (SIRNA) MOLECULAR BEACON FOR THE
DETECTION OF MYCOBACTERIAL INFECTION

by

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ABSTRACT

In latent TB, the ability of *Mycobacterium tuberculosis* to invade and survive within macrophages of the pulmonary granuloma is attributed to protein products of mammalian cell entry (*mce*) 4 operon genes (A-F) that are cholesterol transporters that facilitate the transport of host lipids into the mycobacterium that allows long term survival during chronic infection. Currently, there are no rapid and reliable tests for the detection of latent TB. Therefore, because there is a lack of reliable and efficient tests for the diagnosis of latent TB, we tested the hypothesis that mycobacterial infection can be detected using *mce4* siRNA molecular beacons against *mce4* mRNAs. Because our work showed that the *mce4A* gene of the *mce4* operon conferred infectivity to host *E. coli*, a siRNA molecular beacon was designed against a region of the *mce4A* mRNA that is highly homologous in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. This molecular beacon has a hairpin structure with a stem, 5 nucleotides on either end that are complementary to each other, and a loop which contains 20 nucleotides that are complementary to a region of the target mRNA. Conjugated to the 5’ and 3’ ends of the molecular beacon are the fluorophore TYE 665 and quencher Iowa Black RQ-SP. In the absence of the target mRNA the hairpin structure will predominate and fluorescence will be quenched while in the presence of the target mRNA fluorescence will be induced. Our study shows that the siRNA molecular beacon detects its target in *M. smegmatis* and in macrophages infected with *M. smegmatis* and offers a potential test for detection of mycobacterial infection.
INTRODUCTION

The persistence of latent Tuberculosis (TB) continues to be a significant problem in the world today, more so for third world countries (WHO 2010). Latent TB is characterized by pulmonary granulomas which allow *Mycobacterium tuberculosis* (Mtb) to survive for years without detection (Barry, Boshoff et al. 2009). Most of those with latent TB infection are unaware of their condition until their infection becomes acute which occurs in over 10% of latent TB cases (Mariano 1995). As the world becomes easier to travel, especially through modern modes of transportation, latent TB has become every one’s problem even for highly industrialized countries like the U.S. *M. tuberculosis* survival in granulomas is possible through its ability to synthesize a mammalian cell entry protein, *mce4* (Arruda, Bomfim et al. 1993). This protein is encoded on the *mce4* operon that consists of five genes designated *mce4A-F*. The products of these genes are cholesterol transporters that help transport lipids from the host macrophage into the mycobacterium and allows the mycobacterium to survive for years during chronic infections (Pandey and Sassetti 2008). Currently there are no reliable, rapid tests for the detection of latent TB and because this is asymptomatic it is difficult to treat (Horsburgh and Rubin 2011). Therefore, because there is lack of reliable and efficient tests for the diagnosis of latent TB, 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 detection of mycobacterial infection in macrophages.

More than 80% of tuberculosis (TB) cases in the United States are from reactivation of latent TB infection (Horsburgh and Rubin 2011). The hallmark of pulmonary TB is the granulomas harboring the bacterial infection along with their
draining lymph nodes. Each granuloma has 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). These TB lesions are surrounded by highly vascularized tissue (Ulrichs, Kosmiadi et al. 2005) which enables the targeting of latent Mtb with systemically delivered drugs. The treatment for active or latent TB infection consists of an extended course of antibiotics spanning many months using drugs like isoniazid or rifampicin, which generally carries poor patient compliance rates (Horsburgh and Rubin 2011).

Unfortunately, all available tests today can only strongly suggest the presence of latent TB but cannot confirm it. This is due to low specificity for chest x-ray, low sensitivity for tuberculin skin test in BCG vaccinated individuals, or low sensitivity for interferon gamma release assay in children, immune-compromised persons, and the elderly. Currently, there are no direct imaging methods for locating TB bacteria in the body, which can be critical when trying to identify high risk individuals for prophylactic regimen as well as for identifying extrapulmonary TB sites in HIV co-infected patients. Developing a direct *M. tuberculosis* imaging screening tool for the asymptomatic population is going to be vital in the fight against tuberculosis.

Studies showed that *mce* operons are widely distributed throughout the genus *Mycobacterium*, and *M. smegmatis*, a non-pathogenic mycobacterial species that shares many features with *M. tuberculosis*, possesses a homolog of *mce4* (Altschul, Gish et al. 1990, Haile, Caugant et al. 2002). 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.
Because over 90% of the people infected with Mtb will have latent TB (LTB) infection and 10% will eventually go on to have full-blown active TB at a later stage in their life (Kumar and Robbins 2007), prompt identification and treatment of individuals with LTB is important for the effective control of this disease. Currently, there are no tests available to directly detect the presence of Mtb in an affected individual and assessment of latent TB infection involves an imperfect approach of measuring the host immune response to mycobacterial infection (Horsburgh and Rubin 2011). This testing deficiency can be especially critical when trying to identify high risk individuals for prophylactic regimen as well as identifying extrapulmonary TB sites in HIV co-infected patients (Chakraborty and Chakraborty 2000). Developing a direct Mtb imaging-screening tool for the asymptomatic population along with novel treatment strategies is vital to our fight against tuberculosis.

Molecular beacons (MB) are hairpin shaped single stranded nucleic acid probes that fluoresce only upon hybridization with its target molecule. They have a stem-loop structure, with a fluorophore and a quencher attached to opposite ends. The on/off signals produced by the fluorophore/quencher pair depends on the conformational state of the MB. In the absence of \textit{mce4} mRNA, the stem, which consists of four to seven base pairs, brings the quencher nearby the fluorophore and turns the fluorescence off with high quenching efficiency via Fluorescence Resonance Energy Transfer (FRET). In the presence of the target \textit{mce4} mRNA, the loop region of the molecular beacon hybridizes with the target mRNA and opens up the hairpin structure into a linear structure, thus causing separation of the fluorophore and the quencher which results in increase in fluorescence and identification of the target mycobacterium (Kim, Sohn et al. 2008).
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, rapid detection and effective treatment for TB is critical. To that end, we will test the hypothesis that *Mycobacterial smegmatis* infection can be detected in macrophages using a siRNA molecular beacon against one of the *mce4* operon genes. The findings of these studies will demonstrate the utility of detecting mycobacterial infection using siRNA molecular beacons which can then be easily adapted to detecting infection in animal models and eventually in humans.
METHODS

Mammalian cell culture

Breast cancer MCF7 cells and U937 human monocytic leukemia cells were purchased from the ATCC and were maintained in HQ-DMEM or RPMI medium supplemented with penicillin and streptomycin at 1 unit/ml and enriched with 10% (v/v) fetal bovine serum and 2 mM L-glutamine. Cells were routinely passaged every other day. Cells were cultured in flasks at 37 °C for propagation and in 12 well plates with glass cover slips for differentiation (U937 cells) and infection. U937 cell differentiation was achieved as described previously (Adunyah, Unlap et al. 1992). Briefly, this was done by incubating $1 \times 10^6$ cells with complete RPMI medium supplemented with 4nM PMA. After 2 days of treatment with PMA (differentiation) nonadherent cells were aspirated, coverslips were removed and adherent cells were counted using a haemocytometer with viable cells identified by trypan blue dye exclusion.

Bacterial strains and culture conditions

*M. smegmatis* mc² 155 was purchased from ATCC. *E. coli* Top10, obtained from Invtrogen, was used as the host bacteria for cloning experiments. *M. smegmatis* mc² 155 was grown in Middlebrook 7H9 broth (Difco) with 0.05% Tween 80 and supplemented with OADC (oleic acid, albumin, glucose, catalase supplement). *E. coli* cultures were grown on Lennox L (LB) broth and antibiotics were added as appropriate (50 µg/ml ampicillin for *E. coli* harboring pTrcHis2-TOPO). All cultures were incubated at 37 °C and shaken at 190rpm.
Genomic DNA isolation from *M. smegmatis*

Genomic DNA from *M. smegmatis* was isolated from a 100-ml culture of *M. smegmatis* which was grown to an OD$_{600}$ of 1.6. Approximately 1 x 10$^9$ cells were harvested by centrifugation (12,000g for 1 min) and genomic DNA was extracted using the Axyprep Bacterial Genomic DNA Miniprep Kit (Axygen Biosciences, CA) according to the manufacturer’s protocol. The purified genomic DNA was used for the PCR amplification of specific mce4 operon genes.

**PCR Amplification**

PCR was performed using genomic DNA (50ng) from *M. Smegmatis* and 100ng of gene specific forward and reverse primers in 25µL containing GoTaq® Master Mix (Promega). Genomic DNA from *M. smegmatis* was used along with five sets of primers to amplify a 1.2 Kb *mce4A* fragment (5′-GAGGAGCCATGGATGTGACGGAAACGCAAA-3′ / 5′-GGAAGGAAGCTTGAAGTCGTCCCTTCCGCAAA-3′), a 1.1 Kb *mce4B* fragment (5′-GGAAAGCGATCGTTCTAGATGCACCGCACAGG-3′ / 5′-GCCATTCTCCGAGCACCCTCCC-3′), a 1.0 Kb *mce4C* fragment (5′-TGGCGACTTCCGCTCATCAGT-3′ / 5′-CGGAGATTGGCTAGTCTGC-3′), a 1.4 Kb *mce4D* fragment (5′-TCCGCCCGCACCTCCGAGCAGG-3′ / 5′-GTCCCGCATGACACCACTCATTG-3′) and a 1.7 Kb *mce4F* fragment (5′-CCGTAGATGATCGACCGCTG-3′ / 5′-AGCCTGCCTTGGATCCAGCAT-3′). PCR was carried out using gene specific conditions. Ten microliters of each PCR product was transferred to a separate tube and
mixed with 2 µL of 6x DNA loading dye followed by electrophoresis on a 1% agarose gel at 70v for one hour along with 1kb DNA marker. Samples containing the correct fragment sizes were used for subsequent cloning reactions.

**Cloning of mce4 Genes**

Ligation of each PCR fragment into the vector was achieved by mixing an aliquot (2 µL) of the PCR sample with 1µL of pTrecHis2-TOPO and 2 µL of sterile deionized water (SDW) followed by incubation at room temperature for 5 minutes followed by, transformation and plating on agar plates containing 50µg/ml of ampicillin and incubated overnight at 37 °C. Four colonies were selected and grown over night in L-broth (Difco) containing 50µg/ml ampicillin. Colonies were screened by taking 500µl aliquot of each overnight culture and centrifuging at 13,000xg for 5min, lysed in 100µl of 1XSTE (100mM NaCl, 10mM Tris-HCl, 1mM EDTA) by vigorous vortexing followed by mixing with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), and then vortexed and centrifuged at 13,000xg for 2 minutes. A 20µl aliquot of the aqueous layer (top) was mixed with 4µl of 6X DNA loading dye and resolved by agarose gel. The clones which showed a 4.4 Kb plus the correct sizes of inserts were used for plasmid isolation using the SNAP Midiprep Kit (Invitrogen) according to the manufacturer’s instructions. In order to determine the orientation of each insert, two plasmids representing each gene were sequenced in both directions by SeqWright (SeqWright, Inc). *E. coli* containing each of the mce4 genes (*E.coli*-4A-F) in the correct orientation and the vector alone (*E.coli*-TOPO) were used for functional assays.
**Invasion assay time-course**

In order to determine the virulence that each gene conferred to the host *E. coli*, MCF7 cells were seeded at $2.5 \times 10^5$ cells per well in 12-well plates and incubated for 24hrs. MCF7 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*, *E. coli*-TOPO or *E. coli*-4A-F were added to each well at a multiplicity of infection (MOI) of 10:1 and incubated at 37°C for 3 hours. Cells were washed 3 times with HyQ DMEM media which contained 5% fetal bovine serum (FBS), 1% pen/strep and 100 µg/ml kanamycin to remove extracellular bacteria and lysed after 24, 48 and 72hrs. For lysis, cells were incubated for 10 min in 500µl 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) and incubated at 37°C overnight. Recombinant *E. coli* colonies were counted and the numbers of *E. coli* that survived at 24, 48 and 72hrs post infection was plotted versus time.

**mce4A siRNA molecular beacon**

Our functional assays demonstrated that the mce4A gene conferred virulence to the host *E. coli* which appeared early and was sustained for the entire infection period. Therefore, a molecular beacon siRNA was designed against the mce4A gene. This mce4 siRNA was generated from the *M. smegmatis* genome which spans nucleotides 5960740-5960721 and is homologous to a region in the *M. tuberculosis* H37Rv genome spanning nucleotides 101049-101030. The fluorophore TYE 665 was conjugated to the 5’ end and the quencher, Iowa Black RQ-SP, was conjugated to the 3’ end.
Confocal Imaging

An overnight culture of *M. smegmatis* MC\textsuperscript{2}155 was used to inoculate 7H9 broth at 1/50\textsuperscript{th} volume and grown to an A\textsubscript{600} of 0.3. *Mce4* siRNA molecular beacon or random oligonucleotide molecular beacon was added at 10µM to the *M. smegmatis*, incubated at 37°C for 5hrs and imaged using a confocal microscope.

Detection of *M. smegmatis* in differentiated U937 cells

To determine the ability of the *mce4A* siRNA molecular beacon to detect mycobacterial infection in macrophages, differentiated U937 cells were either not infected or infected at a multiplicity of infection (MOI) of 10:1 bacteria (*M. smegmatis*) per macrophage in a well and incubated at 37°C for 3 hours to allow for phagocytosis to occur. Each well was washed 3 times with RPMI 1640 media which contained 10% fetal bovine serum (FBS), 1% penn/strep and 200 µg/ml amikacin to remove extracellular bacteria. Fresh RPMI media, 3 mls, containing the appropriate antibiotic was added to each well containing the infected or non-infected cells and incubated overnight at 37°C in the presence or absence of 1 or 10µM *mce4A* siRNA molecular beacon. Fluorescence measurements were carried out using a microplate reader, GloMax\textsuperscript{TM} at peak absorbance of 656nm and peak emission wavelength of 665nm.
RESULTS

Cloning of mce4 operon genes

Using gene specific forward and reverse primers, each mce4 gene was PCR amplified from M. smegmatis. Using the pTrcHis2-TOPO cloning system, cloning of the five constituent genes of the mce4 operon of M. smegmatis was accomplished in a timely manner. However, since the genes were amplified through PCR using gene specific primers cloning of each gene into the vector was non directional. Therefore, two positive clones were selected for sequencing to ascertain orientation. The reverse primer for each gene excluded the stop codon in order to express the c-myc and 6xHis tags with each protein product and only clones containing the gene and c-myc and 6xHis tags that were in frame were selected for use in subsequent experiments.

mce4 operon genes confer virulence to E. coli

Which of the five mce4 operon genes is primarily responsible for latent TB infection is not currently known. Therefore, the ability of each of the five mce4 operon genes to confer virulence to the host E. coli was determined by comparing the number of colonies that grew on agar plates following lysis of MCF7 cells which were infected with E. coli expressing the vector (E.coli-TOPO) or each of each of the five mce4 operon genes (E. coli-4A-F). The results showed that while mce4A, B, C, and D genes confer virulence to the host E. coli, mce4A showed virulence that appeared early and was sustained for 24, 48 and 72hr post infection (Fig 1).
**Fig. 1. MCE4 proteins confer virulence to *E.coli*.** *E.coli*-mce4 clones were used to infect MCF7 epithelial cells at an MOI of 10:1 for 2 hours. The level of infection was assessed by comparing bacterial colonies at 24, 48 and 72 hours post-infection; n=3.
The design of a $mce4A$ siRNA molecular beacon

Because $mce4A$ conferred virulence to the host E.coli which appeared early and was sustained for the entire 72hr period, a siRNA molecular beacon was designed against this gene (Fig 2). This siRNA molecular beacon consists of 20 nucleotides which are complementary to a region of the $mce4A$ mRNA and 5 nucleotides on the 5’ end and another 5 nucleotides on the 3’end. The 5 nucleotides on either end of the structure are complementary to each other such that they form a double stranded stem for the molecular beacon. Also conjugated to the 5’ and 3’ ends of the molecular beacon were the fluorophore TYE 665 and the quencher Iowa Black RQ-SP, respectively. The rationale in the design was that in the absence of its target mRNA, the $mce4A$ molecular beacon will be in the form of a hair loop structure and fluorescence will be quenched. In the presence of the target mRNA, however, the molecular beacon will open up and bind with the 21 complementary nucleotides to the mRNA. This will position the fluorophore and the quencher apart such that fluorescence will not be quenched.

The $mce4A$ siRNA molecular beacon interacts with its target in $M. smegmatis$

Once the $mce4A$ siRNA was designed, it was necessary to demonstrate that it would interact with its $mce4A$ mRNA target in $M. smegmatis$. $Mce4$ siRNA molecular beacon or random oligonucleotide molecular beacon was added at 10µM to the $M. smegmatis$, incubated at 37°C for 5hrs and imaged using a confocal microscope. Confocal microscopy imaging demonstrated that the siRNA but not random oligonucleotide molecular beacon interacted with its target mRNA in $M. smegmatis$ (Fig 3).
Fig. 2. The sequence of a siRNA molecular beacon against mce 4A. A molecular beacon was generated against mce4 mRNA from the non-virulent Mycobacterium, *M. smegmatis* MC\(^2\)155 (Ms, A). This mce4 siRNA was generated from the Ms genome which spans nucleotides 5960740-5960721 (B) which is homologous to a region in the *M. tuberculosis* H\(_{37}\)Rv genome spanning nucleotides 101049-101030 (C). The fluorophore TYE 665 was conjugated to the 5’ end and the quencher, Iowa Black RQ-SP, was conjugated to the 3’ end. A random molecular beacon was also generated as a negative control that does not have any match in the entire mycobacterial or human genome (D).
Fig. 3. *Mce4A* siRNA molecular beacon interacts with its target in *M. smegmatis*.

An overnight culture of *M. smegmatis* MC^2^155 was used to inoculate 7H9 broth and grown to an A_{600} of 0.3. *Mce4* siRNA molecular beacon or random oligonucleotide molecular beacon was added at 10µM to the *M. smegmatis*, incubated at 37°C for 5hrs and imaged using a confocal microscope.
The mce4A siRNA molecular beacon interacts with its target in *M. smegmatis*

The designed *mce4A* siRNA molecular beacon was used for the detection of *M. smegmatis* infection in infected U937 cells. Infected and noninfected U937 cells were transfected with 1 or 10µM of the siRNA or a random oligonucleotide molecular beacon, incubated overnight followed by fluorescence measurement using a GloMax®-Multi microplate reader. Our results (Fig 4) demonstrated that baseline fluorescence was observed in noninfected cells not transfected or transfected with 10µM of the siRNA or random oligonucleotide molecular beacon and in infected cells which were not transfected or transfected with 1 or 10µM of the random oligonucleotide molecular beacon. Cells infected with *M. smegmatis* and transfected with the siRNA molecular beacon showed significant levels of fluorescence which were above baseline levels. Comparison of fluorescence between baseline levels and fluorescence levels in infected cells transfected with 1 or 10µM of the *mce4A* siRNA molecular beacon using Analysis of Variance (ANOVA) indicated significance at $p \leq 0.001$. 
Figure 4: Fluorescence measurement in *M. smegmatis* infected U937 cells. Non-infected or Ms-infected U937 macrophages were either not transfected (pink) or transfected with random oligonucleotide (purple) or *mce4A* siRNA (green) molecular beacon at 1 or 10µM followed by fluorescence measurement after 3hr incubation. The effect of molecular beacon transfection on fluorescence intensity was compared between transfected and non-transfected samples using ANOVA. n=32; *, +p≤0.001
DISCUSSION

The use of molecular beacons for in vivo detection of mRNA is gaining popularity. These small hairpin structures are stable in cellular environments and bind to their target mRNAs with a high degree of specificity. They can be easily modified by the addition of dyes and quenchers in order to enhance their utility in detection protocols. Molecular beacons have been used in a number of areas including detection of specific mRNA targets in vivo (Bratu, Catrina et al. 2011) and in cervical living cells including cancer cells (Rhee, Santangelo et al. 2008, Xue, An et al. 2011), identification of allelic variance for drug resistance (Balashov, Gardiner et al. 2005), and detection of organisms for diagnostic tests (Warren, Liao et al. 2004, Balashov, Gardiner et al. 2005, Santangelo, Nitin et al. 2006, Morandi, Ferrari et al. 2007).

Small interfering RNA (siRNA) technology has been used to inhibit transcription for hepatitis G virus (Cao, Ren et al. 2005), influenza virus (Ge, McManus et al. 2003), picornavirus (Lim, Yuan et al. 2008), and Trypanosoma brucei (Zhang, Guo et al. 2007). The potential combination of the two technologies, molecular beacon and siRNA, in the health care industry is tremendous. Molecular beacon siRNA have been used for knocking down the expression of telomerase mRNA in human breast cancer cells (Zhou, Mao et al. 2011), BMP4 mRNA in hedgehog signaling (Rhee, Santangelo et al. 2008), and aromatase mRNA in breast cancer cells (Zhou, Mao et al. 2011). Thus, siRNA can be designed in the form of molecular beacons in order to be used in detection and therapeutics.
This study was necessitated by three things, 1) latent TB continues to be a problem not only for third world countries but also for industrialized countries like the U.S., 2) because the mycobacterium growth rate is so slow rapid and specific diagnostic 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 detect and treat latent TB, this study was conducted in order to test the hypothesis that a molecular beacon siRNA designed against the \textit{mce4} operon, which has been shown to be responsible for latent TB infection (Arruda, Bomfim et al. 1993, Saini, Sharma et al. 2008), especially \textit{mce4A} (Xu, Li et al. 2007, Saini, Sharma et al. 2008), could be used for detection and attenuation of mycobacterial infection. This hypothesis was tested in MCF7 breast cancer cells and in differentiated U937 cells.

In order to design a molecular beacon siRNA, it was necessary to determine which of the \textit{mce4} operon genes confer the highest degree of virulence to host \textit{E. coli}. Because the \textit{M. tuberculosis} has a slow growth rate and there is high degree of homology between \textit{mce4} operons of \textit{M. tuberculosis} and other mycobacteria (Haile, Caugant et al. 2002), the \textit{mce4} operon of the rapid growing \textit{M. smegmatis} was selected. Using gene specific primers with the reverse primer for each set excluding the termination codon, \textit{mce4A-F} were PCR amplified, cloned into the prokaryotic expression vector pTrcHis2-TOPO and stably expressed in \textit{E. coli}. Western blot assays using cmyc and 6xHis monoclonal antibodies showed that the proteins were expressed in host \textit{E.coli}. Invasion assays in MCF7 breast cancer cells (Fig 1) showed that \textit{mce4A-F} conferred virulence to the host \textit{E.coli}. However \textit{mce4A} conferred virulence which was high from the beginning.
and was sustained during the entire invasion period (72hr). Therefore mce4A was selected to be the target for a molecular beacon antisense RNA (Fig 2).

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 20 nucleotides that are complementary to a region of the target mce4A mRNA and conjugated to the 5’ and 3’ ends of this molecular beacon are the fluorophore TYE 665 and quencher Iowa Black RQ-SP, respectively. The double stranded stem facilitates cytosolic localization of the siRNA (Chen, Davydenko et al. 2010) and in the presence of the target mce4A mRNA the siRNA molecular beacon will bind and fluorescence intensity will increase, since the fluorophore and the quencher are separated, and degradation of the target mRNA will be induced.

This molecular beacon design combines both detection and therapeutic(Chang, Zhu et al. 2007, Rhee, Santangelo et al. 2008, Zhou, Mao et al. 2011). The rationale is 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 20 nucleotide loop will compete with the 5 nucleotide stem for hybridization to their targets, the loop to the mce4A mRNA and the stem to its complementary pair on the opposite end of the molecular beacon. The hybridization of the loop, based on the number of nucleotides (20 versus 5), to its target will be greater than that of the strands for the stem. Hybridization of the loop to the mce4A mRNA will separate the fluorophore from the quencher which will induce fluorescence (detection) and degradation (therapeutic) of the mRNA. Because the mycobacterium utilizes the product of mce4A for survival on cholesterol for
carbon and energy source (Xu, Li et al. 2007, Saini, Sharma et al. 2008, Senaratne, Sidders et al. 2008, Miner, Chang et al. 2009), degradation of the mce4A mRNA will lead to reduced survival. This study tested the ability of the mce4A siRNA to detect its target mce4A mRNA in M. smegmatis and in macrophages infected with M. smegmatis. The results show that the molecular beacon siRNA detects its target in M. smegmatis and in macrophages infected with M. smegmatis. Thus, a molecular beacon can be designed against one of the mce4 operon genes in M. smegmatis that facilitates the detection of mycobacterial infection in macrophages. Current tests are being carried out to test the ability of this siRNA molecular beacon to not only detect but also attenuate mycobacterial infection in macrophages.
REFERENCES


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

by

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ABSTRACT

The ability of pathogen *Mycobacterium tuberculosis* (MTB) to invade and survive within macrophages of the pulmonary granuloma is attributed to the product of the mammalian cell entry (mce) operon whose gene, *mce4A*, encodes a cholesterol transporter that helps transport host lipids into the bacterium that allows the bacterium to survive for years during chronic infection. Here, we proposed and tested the hypothesis that a *mce4A* siRNA molecular beacon construct against the *mce4A* mRNA 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 against the *mce4A* mRNA for 24hrs. This was followed by infection with either *E. coli-4A* or *M. smegmatis* for 3hrs followed by incubation for 0, 3, 6, 24, and 48hrs. The cells were lysed and the lysates were plated on LB agar plates containing ampicillin (100µg/ml) 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 hours 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 hours by 94.8%, 70.3%, 98.9%, and 93.4%, respectively. In conclusion, a *mce4A siRNA* molecular beacon construct 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 2010, Shea, Kammerer et al. 2014). LTB is characterized by pulmonary granulomas which allow Mycobacterium tuberculosis (Mtb) to survive for years without detection (Barry, Boshoff et al. 2009). 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, Rahman et al. 2013). These TB lesions are surrounded by highly vascularized tissue (Ulrichs, Kosmiadi 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, Painter 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, Bomfim et al. 1993). These proteins are encoded on the *mce*4 operon that consists of five genes designated *mce*4A-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 *mce*4 (Altschul, Gish et al. 1990, Haile, Caugant et al. 2002, Rathor, Chandolia 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, Smith et al. 2012).

Since about 90% of the TB patients develop LTB and 10% go on to have acute TB (Kumar and Robbins 2007), 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, Sternberg 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 (Van der Geize, Yam et al. 2007, Pandey and Sassetti 2008, Miner, Chang et al. 2009).

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, Bolus 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 of penicillin and streptomycin in a 5% CO2 humidified atmosphere. Cells were passaged at a density of approximately $2 \times 10^6$ cells/ml 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, Unlap et al. 1992). Briefly, cells were counted using a hemocytometer and $1 \times 10^6$ cells were incubated with complete RPMI medium containing 4nM PMA. After 48 hours 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 $A_{600}$ 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 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 hours. 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 5x DNA ligase buffer, and 1µL of T4 DNA ligase followed by incubation at room temperature for 2 hours. The ligation mix was used to transform E. coli DH5α cells followed by plating on LB agar plates containing 50µg/mL kanamycin and overnight incubation at 37°C. Ten colonies were selected and grown overnight in LB broth containing 50µg/mL 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 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 minutes 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% CO2 incubator. Viral supernatants were harvested after 48hrs 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 hours in a 12 well plate containing rattail collagen coated coverslips at 1 x 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 hours. Cells were washed in 1xPBS, fixed for 15 minutes 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 60X 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 24hrs, followed by infection with either E. coli-4A or M. smegmatis for 3 hrs followed by incubation for 0, 3, 6, 24, and 48hrs. 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, 5.2µg 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 three hours. 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 minutes each using 100 ml volumes of PBS/0.1% Tween 20. The membrane was probed for 2hrs 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 $2.5 \times 10^5$ cells per well in 12-well plates and incubated for 24hrs. Seeded cells were either transduced or not-transduced with $pLenti$-siRNA-GFP construct at a MOI of 5:1 for 24 hours. 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 hours. Cells were washed 3 times with HyQ DMEM media which contained 5% fetal bovine serum (FBS), 1% pen/strep and 100 µg/ml kanamycin to remove extracellular bacteria and lysed after 0, 3, 6, 24, and 48hrs. For lysis, cells were incubated for 10 min in 500µl 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) 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 48hrs post infection was plotted versus time. Infection levels at 3,6,24, and 48hrs were compared to that of base line level (0hr) 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 \(\leq 0.05\) is considered significant.

3. RESULTS

3.1 Cloning of mce4A siRNA in piLenti-GFP vector

A region of the mce4A gene in \(M.\ smegmatis\ MC1552\) 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 EcoRV 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 48hrs of transfection were filtered through 0.45-μm PVDF syringe filter, concentrated using Ultra-Pure® (Applied Biological Materials, Inc.) lentivirus urification...
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.

### 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).

### 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 24hrs, followed by infection with either *E. coli-4A* or *M. smegmatis* for 3hrs followed by incubation for 0, 3, 6, 24, and 48hrs. 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 24hrs followed by infection with *E.coli-
4A or *M. smegmatis* for 3 hours, and incubated for 0, 3, 6, 24, and 48hrs. 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 (100µg/ml) 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 48hrs against that at 0hr 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 48hrs against that at 0hr and was found to be 94.8%, 70.3%, 98.9%, and 93.4%, respectively. 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).
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 rattail collagen coated coverslips for 48 hours with 4nM PMA and were either not transduced (A) or transduced (B) with \textit{piLenti-siRNA-GFP} construct at a MOI of 5:1 for 24 hours. Cells were washed in 1xPBS, fixed for 15 minutes with 4\% PFA and mounted on slides using Vectashield® medium containing propidium iodide followed by fluorescence imaging using a confocal microscope.
Fig. 3. The GFP is immunodetected in transduced U937 cells infected with *E.coli-4A* or *M. smegmatis*. U937 macrophages were differentiated for 48 hours with 4nM PMA in 12-well plates at $1 \times 10^6$ cells per well and transduced with *piLenti-siRNA-GFP* construct at a MOI of 5:1 for 24 hours. Transduced U937 cells were infected with IPTG-induced log phase cultures of recombinant *E. coli-4A* or *M. smegmatis MC^2155* at a MOI of 10:1 for 3 hours, followed by extensive washing and incubation for 0, 3, 6, 24, and 48 hours. 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 kDa.
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 hours 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 hours, followed by extensive washing and incubation for 0, 3, 6, 24, and 48 hours. Cells were washed extensively in 1x 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) 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 48hrs post infection are compared with those at 0hr using ANOVA. n=4; *P*≤0.05; **P*≤0.01
DISCUSSION


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, Sohn et al. 2008, Hong, Zhang et al. 2010) (Ilieva, Della Vedova et al. 2013). Small interfering RNA (siRNA) technology has been used to inhibit transcription of hepatitis G virus (Cao, Ren et al. 2005), influenza virus (Ge, McManus et al. 2003), picorna virus (Lim, Yuan et al. 2008), and trypanosma brucei (Zhang, Guo et al. 2007). SiRNA molecular beacons have been used successfully in the detection and knockdown of telomerase expression in human breast cancer cells (Chang, Zhu et al. 2007), BMP4 mRNA in hedgehog signaling (Rhee, Santangelo et al. 2008), and aromatase mRNA in breast cancer cells (Zhou, Mao et al. 2011). SiRNA technology has been tested successfully for silencing genes in M. tuberculosis (Harth and Horwitz 1999, Li, Chen et al. 2007) and for inhibiting bacterial growth in human macrophages infected with M. tuberculosis (Harth, Zamecnik 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, Unlap 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, Bomfim et al. 1993, Saini, Sharma et al. 2008), especially *mce4A* (Xu, Li et al. 2007, Saini, Sharma et al. 2008), 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, Unlap 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, Caugant 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, Davydenko 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 hours post infection (fig. 2 and fig. 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, Sternberg et al. 2012). Because the mycobacterium utilizes the
product of mce4A for survival on cholesterol for carbon and energy source (Xu, Li et al. 2007, Saini, Sharma et al. 2008, Senaratne, Sidders et al. 2008, Miner, Chang et al. 2009), 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.

4. 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.
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A SHORT INTERFERING RNA (siRNA) MOLECULAR BEACON FOR THE
ATTENUATION OF MCE4A mRNA IN MACROPHAGES

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The ability of *Mtb* to invade and survive within macrophages of the pulmonary granuloma is attributed to the product of the mammalian cell entry (*mce*) genes whose operon, *mce4*, encodes a cholesterol transporter that helps transport host lipids into the bacterium allowing it to survive for years during chronic infection. Currently, there are no effective treatment against latent TB and the lack of treatment leads to persistent latent infection which can become active at a later stage. Therefore, because there is a lack of effective and efficient treatment for latent TB, we tested the hypothesis that mycobacterial infection in macrophages can be eradicated using siRNA molecular beacons against the mRNA of the most infective gene in the *mce4* operon. This hypothesis was tested in U937 cells transduced with a siRNA molecular beacon construct against the most infective *mce4* gene from *Mycobacterium smegmatis* and which were infected with *E. coli* stably expressing this most infective *mce4* gene (*E. coli4*), followed by RTPCR and cell invasion assays to determine effect on *mce4* mRNA and infection levels, respectively. Our results show that a molecular beacon can be designed against one of the *mce4* operon genes in *M. smegmatis* that facilitates the attenuation of mycobacterial *mce4A* mRNA responsible for mediating latent infection in macrophages. The findings of these studies demonstrate proof of concept that latent TB infection can be rapidly and effectively eradicated *in vitro*. 

**ABSTRACT**
INTRODUCTION

Tuberculosis is one of the leading causes of infectious disease mediated deaths in the world today (Eurosurveillance editorial, 2013; Shea et al., 2014). The World Health Organization estimates that one-third of the world population has latent tuberculosis which can be activated upon the right conditions in immunocompromised persons (WHO, 2010). Tuberculosis is mediated by the pathogen *Mycobacterium tuberculosis* (*Mtb*) and the active form of the disease is transmitted from person to person through aerosolized cough droplets. Once entering the host, the ability of this versatile pathogen to survive decades within the body of the host by subverting the host immune defenses is of continued intrigue and fascination to scientists worldwide. Currently, it is of general consensus that the bacteria survives within the host macrophages in the granulomas of the lung (Ulrichs and Kaufmann, 2006). The precise mechanism of how the bacteria is able to achieve this long term dormancy leading to latent tuberculosis is still unknown, however, recent advances in mycobacterial molecular biology have shed some light into these processes.

Generally, when a host animal encounters invading non-pathogenic microbial organisms, the microbes are internalized by the host macrophages creating a phagosome containing a hostile environment of reactive oxygen species and reactive nitrogen species against the microbe (Koul et al., 2004). The phagosome then goes on to mature and fuse with the organelles of the endocytic pathway, thereby acquiring surface molecular markers which leads to the acidification of the phagosome to pH 5 as well as gaining hydrolytic enzymes that digest the invading microbe (Chan et al., 1992; Denis, 1991). However, the mycobacterial pathogen is successful in subverting this host defense
machinery by preventing the phagolysosome formation and prevents the maturation of the mycobacterial phagosomes by not acidifying more than pH 6-6.5 due to depletion of vesicular proton-ATPase at the phagosomal membrane, thereby creating a hospitable environment for itself within the phagosome (Armstrong and Hart, 1971; Fratti et al., 2003). The mycobacterial genes that mediate these adaptive responses are largely yet to be determined.

Over the recent years, it was shown that a DNA fragment from \textit{M. tuberculosis} cloned into \textit{E. coli} could mediate the latter’s entry and survival in mammalian cells (Arruda et al., 1993) and was named as the mammalian cell entry (\textit{mce}) operon. The \textit{mce} operon genes have been shown to be important in the invasion of the mammalian host cell by the mycobacterium and for establishing a persistent infection both in vivo and in mouse models (Flesselles et al., 1999; Senaratne et al., 2008). The analysis of the complete gene sequence of \textit{M. tuberculosis} in 1998 (Cole et al., 1998) showed that the \textit{mce} operon is composed of a group of four homologous \textit{mce} operons (\textit{mce1}, \textit{mce2}, \textit{mce3}, and \textit{mce4}). It was found that all the constituent \textit{mce} genes in the four operons were arranged in an identical manner. Each of the operon contained eight genes, of which two genes preceding the \textit{mce} genes encoded for integral membrane proteins and the six \textit{mce} genes potentially encoding exported proteins (secreted or surface-exposed) thought to be important for the entry and survival of the pathogen in the mammalian cells (Cole et al., 1998). Deletion mutants of one or more of these operons have shown to provide varying degrees of attenuation for the mycobacterial infection both in-vivo and in mice. The deletion of \textit{mce1} operon, however, has showed an increase in virulence for the mycobacteria (Shimono et al., 2003; Uchida et al., 2007) whereas the deletion of \textit{mce2}
operon did not have any effects on the virulence of *Mtb* in either macrophages or animal models. The deletion of *mce2* operon did on the other hand enhance the host’s immune response to *Mtb* infection which led to improved survival in animal models (Marjanovic et al., 2010). Studies have shown that deletion of *mce* operons 3 and 4 attenuated *Mtb* virulence in infected macrophages, and animal models of *mce4* operon deletion showed the greatest effects on *Mtb* virulence (Senaratne et al., 2008; Joshi et al., 2006).

Our current knowledge of the role of each constituent gene in the four operons is limited. *yrbEA* and *yrbEB* genes of the *mce* operon has been proposed to encode the transmembrane permease subunits of the ABC transporters (Casali and Riley, 2007). The 6 *mce* genes in the operon have been thought to encode the outer membrane complex connecting to the inner membrane ABC transporters (Song et al., 2008). Apart from its possible role in mediating persistence of host infection, it is possible that at least some of the MCE4 proteins form an outer membrane channel that enables cholesterol to enter the cell, thereby enabling the mycobacterium to uptake host lipids vital for its survival during the prolonged latent infection (Niederweis et al., 2010). The *mce4* operon is thought to encode a cholesterol transport system that enables the mycobacterium to derive both carbon and energy from the host membrane lipids and to mediate its long term survival (Pandey and Sassetti, 2008).

The *mce* operons are widely seen throughout the genus Mycobacterium and a homolog of *mce4* has been confirmed in the mycobacterial species *M. smegmatis* (Haile et al., 2002; Rathor et al., 2013). Even though *M. smegmatis* is non-pathogenic, previous studies have shown that it can survive and multiply within macrophages in a pathogen-like manner by manipulating the host cell during initial stages by delaying phagosomal
acidification and recruitment of V-ATPase, thus making it a suitable model to study mce4 operon mediated intracellular mycobacterial survival (Kuehnel et al., 2001; Anes et al., 2006).

The mce4A gene is the first among the six mce genes in the mce4 operon that has been studied the most. Saini et al showed that the MCE4A protein of mce4 operon is not only important for host cell invasion but also for survival of the Mtb pathogen in human macrophages (Saini et al., 2008). Our previous studies involving recombinant E.coli expressing various mce4 operon genes showed that mce4A conferred the greatest degree of virulence to the E. coli host (George et al., 2012).

In order to conduct experiments towards the attenuation of mce4A mRNA in macrophages, we first cloned each of the mce4 operon genes, mce4A, mce4B, mce4C, mce4D, and mce4F of M. smegmatis into Top10 E. coli. This was done using gene specific primers with the reverse primer for each set excluding the termination codon and each of the mce4A-F genes were PCR amplified, cloned into the prokaryotic expression vector pTrcHis2-TOPO, followed by stable expression in E. coli. The infectivity of individual genes were confirmed using cell invasion assay and we designed a mce4A short-interfering RNA (siRNA) molecular beacon against the M. smegmatis mce4A mRNA. This antisense molecular beacon had a stem loop structure, with the 5-base pair double stranded stem consisting of nucleotides that are complimentary to each other and a 29 nucleotide loop that is complimentary to a region of the target mce4A mRNA. The double stranded stem facilitates the efficient cytosolic localization of the siRNA (Chen et al., 2010) in the bacterium within the infected macrophage and in the presence of the target mce4A mRNA, the siRNA molecular beacon binds and degrades the target mRNA.
using the bacterial interference machinery (Harth et al., 2002; van der Oost and Brouns, 2009; Wiedenheft et al., 2012). Previously, we were able to show that our mce4A siRNA molecular beacon was able to successfully localize mycobacterial infection in macrophages (George et al., 2012). We then 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 mce4A gene (E. Coli-4A) or M. smegmatis and our results showed that the mce4A siRNA was able to attenuate both E. coli-4A and M. smegmatis infection in macrophages (George et al., 2014).

Our present experiments were designed to further test the hypothesis that the mce4A siRNA molecular beacon can attenuate the mce4A mRNA levels in E. coli expressing mce4A gene within infected macrophages.
METHODS

Mammalian cell culture:

Human MCF7 cells are grown in HyQ DMEM (Fisher Scientific) supplemented with 5% Fetalclone III (Fisher Scientific) and 2mM L-glutamine, 82 units/ml penicillin, and 82 μg/ml streptomycin (Life Technologies, Inc.) at 37°C. The media is routinely changed every other day by aspirating old media followed by replacement with fresh media. MCF7 cells are cultured in flasks at 37 °C in a 5% CO₂ humidified incubator for propagation and in 12 well plates for infection which is carried out for 24hrs.

Macrophage cell culture:

U937 human monocytic cells were obtained from the American Type Tissue Culture bank (ATCC) and were maintained in Dulbecco's Modified Eagle's medium (DMEM) with 10% iron-supplemented calf serum and 50 U/ml of penicillin and streptomycin in a 5% CO₂ humidified atmosphere. Cells are passaged at a density of approximately 2 × 10⁶ cells/ml every other day.

Bacterial strains and culture conditions:

*M. smegmatis* mc² 155 was purchased from ATCC. *E. coli* Top10, obtained from Invitrogen, was used as the host strain for cloning experiments. *M. tuberculosis H₃₇Rv* genomic DNA was purchased from ATCC. *M. smegmatis* mc² 155 was grown in Middlebrook 7H9 broth (Difco) with 0.05% Tween 80 and supplemented with OADC (oleic acid, albumin, glucose, catalase supplement). *E. coli* TOP10/ DH5α cultures are grown on Lennox L (LB) broth with incubation at 37 °C and shaking at 190rpm.
Recombinant *E. coli* cultures harboring pTrcHis2-TOPO clones are grown on Lennox L (LB) broth containing 50 µg/ml ampicillin and the expression of the recombinant protein was induced as follows: A single colony of recombinant *E. Coli* was inoculated into 2ml of LB broth containing 50 µg/ml ampicillin and grown overnight at 37 °C with constant shaking at 190rpm. The next day, 10ml of LB medium containing 50 µg/ml ampicillin was inoculated with 1/50th volume of overnight recombinant *E. Coli* culture and grown to mid-log phase (OD600 = 0.6) at 37 °C with shaking at 190rpm. To induce the recombinant protein expression, isopropyl-β-D-thio-galactoside (IPTG) was added to the log-phase cultures at a final concentration of 1mM and grown at 37 °C with shaking for 5 hours.

**Genomic DNA isolation from M. smegmatis:**

Genomic DNA from *M. smegmatis* was isolated from a 100-ml culture grown to an OD<sub>600</sub> of 0.6. Approximately 1 x 10<sup>9</sup> cells are harvested by centrifugation (12,000g for 1 min) and genomic DNA was extracted using the Axyprep Bacterial Genomic DNA Miniprep Kit (Axygen Biosciences, CA), according to the manufacturer’s protocol. The purified genomic DNA was used for PCR amplification of specific *mce4* operon genes.

**PCR Amplification:**

In order to generate each *mce4* operon genes from *M. smegmatis*, PCR was performed using the genomic DNA (50ng) from *M. Smegmatis MC<sup>2</sup>155 and 100ng of gene specific forward and reverse primers in 25µL containing 2X GoTaq® Master Mix (Promega). Genomic DNA from M. smegmatis was used along with five sets of primers to amplify a 1.2Kb *mce4A* fragment (5′-
GAGGAGCCATGGATGTCGAACGGAAACGCCAAA-3’ / 5’

GGAAAGGAAGCTTGAAGTCGTCCCTTTCCGCGAA-3’), a 1.1Kb mce4B fragment
(5’-GGAAAGCGATCGTTCTAGATGCACCGCGACAGG-3’ / 5’-GCCATTCTCCGAGCACCTCCC-3’), a 1.0Kb mce4C fragment (5’-TGGCGACTTCGCCCTCAGAT-3’ / 5’-CGGAGAATGGGTAGTCTGCGC-3’), a 1.4Kb mce4D fragment (5’-TCCGCCGCACCTGCCGGAGC-3’ / 5’-GTCGCCATGACACATTCGAAT-3’), and a 1.7Kb mce4F fragment (5’-CCGTAGATGATCGACCGGCTG-3’ / 5’-AGCCTGCCTTGGATCCAGCAT-3’).

PCR was carried out using gene specific conditions for mce4A (94°C for 2 min and 30 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min followed by a long extension time of 10 min at 72°C), mce4B (94°C for 2 min, 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min followed by a long extension time of 10 min at 72°C), mce4C (94°C for 2 min, 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min followed by a long extension time of 10 min at 72°C), mce4D (94°C for 2 min, 30 cycles at 94°C for 1 min, 50°C for 1 min, 65°C for 1 min and 72°C for 1 min followed by a long extension time of 10 min at 72°C), and mce4F (94°C for 2 min, 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min followed by a long extension time of 10 min at 72°C). Ten microliters of each PCR products was transferred to separate tubes and mixed with 2 µL of 6x DNA loading dye followed by electrophoresis on a 1% agarose gel at 70v for one hour along with 1kb DNA marker.
Cloning of mce4 genes:

Ligation of each PCR fragment into the vector was achieved by mixing an aliquot (2 µL) of the PCR sample with 1µL of pTrcHis2-TOPO and 2 µL of sterile deionized water (SDW) followed by incubation at room temperature for 5 minutes. Transformation was performed by mixing 2 µL of the ligation mix with 30 µL of competent E. coli followed by incubation on ice for 30 minutes. The transformation mix is heat-shocked at 42 °C for 30 seconds and immediately transferred to ice for 2 minutes followed by addition of 250 µL of SOC media and incubation at 37 °C for 1 hour with constant shaking. Two aliquots, 10 and 100µL, of the transformation mix are plated out on agar plates containing 50µg/ml of ampicillin and incubated overnight at 37 °C. Four colonies are selected and grown over night in L-broth (Difco) containing 50µg/ml ampicillin. Colonies are screened by taking 500µl aliquot of each overnight culture and centrifuging at 13,000xg for 5min, lysed in 100µl of 1XSTE (100mM NaCl, 10mM Tris-HCl, 1mM EDTA) by vigorous vortexing followed by mixing with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The samples are vortexed and centrifuged at 13,000xg for 2 minutes. A 20µl aliquot of the aqueous layer (top) was mixed with 4µl of 6X DNA loading dye and resolved by agarose gel electrophoresis at 70 volts on a 1% agarose gel against supercoiled DNA markers. The clones which showed a 4.4Kb + the correct size of insert are used for plasmid isolation using the SNAP Midiprep Kit (Invitrogen) according to the manufacturer’s instructions. In order to determine the orientation of each insert, each plasmid was sequenced in both directions by SeqWright (SeqWright, Inc). E. coli containing the mce4 genes in the correct orientation and an E. coli containing the vector are used for functional assays.
MCE4A-F Western blot analyses:

Once the PCR product of interest is cloned into pTrcHis2-TOPO and the construct transformed into E. coli, the expression of the recombinant MCE4 protein with 6xHis/ c-myc tag is induced with isopropyl β-thiogalactoside (IPTG). E. coli carrying the empty vector served as the negative control for the Western blots. IPTG is added to the log-phase cultures at a final concentration of 1mM and grown at 37 °C with shaking for 5 hours. Bacterial cultures are pelleted at 5000 rpm for 10 minutes. The supernatant is aspirated and the bacterial pellet is suspended in 250 microliters of lysis buffer (10 mM Tris-HCl (pH 7.4), 140 mM NaCl, 1% (v/v) Triton X-100, 10 mM EDTA) and vortexed vigorously in a microcentrifuge tube. The sample was centrifuged at 14,000xg and the supernatant was transferred to a new microcentrifuge tube. The MCE4A protein from E. coli-Ms4 is detected using antibodies against 6XHis or c-myc by Western blot as previously described (Unlap and Jope, 1997a). Specifically, 50µg of protein fractions were 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 was rinsed in 10 mM 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 one hour. Following rinsing with PBS the membrane was slowly shaken overnight with a 1:5000 dilution of primary antibody (mouse anti-His or anti-c-myc monoclonal antibody (Invitrogen, Inc)). The blot was rinsed twice with PBS/0.1% Tween 20 and then washed four times for 5 minutes each using 100 ml volumes of PBS/0.1% Tween 20. The membrane was probed overnight at 4°C with a 1:1000 dilution of horseradish peroxidase
(HRP) conjugated goat anti-mouse IgG (Invitrogen) in 10 ml Blotto. Visualization of 6xHis/c-myc labeled MCE4 recombinant proteins 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 immediately exposed to X-ray film (Hyperfilm-ECL, GE) and developed.

*E. coli-4A-F invasion assay time-course:*

MCF7 cells are seeded at 2.5 × 10⁵ cells per well in 12-well plates and incubated for 24hrs. IPTG is added to log-phase cultures of *E. coli-Ms4* or *E.Coli-Mtb4 cultures* at a final concentration of 1 mM and grown at 37°C with shaking for 5 hours. MCF7 cells are incubated in fresh medium (HyQ DMEM supplemented with 5% FCS and 2 mM L-glutamine) at 37°C for 30 min and *E. coli* or *E.coli*-pTrc are added to each well at a multiplicity of infection (MOI) of 10:1 and incubated at 37°C for 3 hours to allow infection to occur. Wells are washed 3 times with HyQ DMEM media which contained 5% Fetaclone III, 1% penn/strep and 100 µg/ml kanamycin to remove extracellular bacteria. Cells are lysed after 24, 48 and 72hrs after infection and incubation at 37°C. For lysis, cells are incubated for 10 min in 500µl of lysis buffer (0.1% Triton X-100 in PBS, pH 7.4) and mixed using a micropippette. The lysate is plated on LB agar plates containing ampicillin (100µg/ml) and incubated at 37°C overnight. Recombinant *E. coli* colonies are counted and plotted as a function of time. The results are compared using ANOVA.
Cloning of siRNA in \textit{piLenti-GFP}

The siRNA target sequence against \textit{mce4A} gene was selected using antisense design software from Integrated DNA Technologies (IDT). A region of the target sequence covering nucleotides 5960719-5960747 of \textit{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 \textit{Bbs I} site of the \textit{iLenti-GFP} vector (5’AAAATCGGCAGGCTCTCGGGATAGGTGTATCCC 3’; 3’AGCCGTCCGAGAGCCCTATCCACATAGGGGAAA 5’) (Applied Biological Materials, Inc). This sequence was designed so that when transcribed, the transcript will generate a siRNA with complimentary 5’ and 3’ ends that forms a hairpin structure. This double stranded (ds) fragment was then ligated into the \textit{iLenti-GFP} vector by mixing an aliquot (3µL) of the ds sequence, 4µL of \textit{Bbs I} linearized \textit{piLenti-GFP} vector, 2µL of 5x DNA ligase buffer, and 1µL of T4 DNA ligase, and then incubated at room temperature for 2 hours. The ligation mix was used to transform \textit{E. coli DH5a} cells and was plated on LB agar plates containing 50µg/mL kanamycin, followed by overnight incubation at 37°C. Ten selected colonies were grown overnight in LB broth containing 50µg/mL kanamycin. Plasmid isolation from each of the colonies was performed using the SNAP Midiprep kit (Invitrogen) according to the manufacturer’s protocol and the plasmids were screened by \textit{EcoR V} digestion to identify clones with the correct insert size and orientation. \textit{E. coli} possessing the insert with the correct size and orientation was used for lentivirus production.
Generation of *piLenti-siRNA-GFP* phage

The *piLenti-siRNA-GFP* phage was generated using 293T cells. 293T cells were grown in a 10cm tissue culture plate at 90% confluency. A transfection mix consisting of the following was premade using 2mL complete medium supplemented with 0.1mM MEM Non-Essential Amino Acids, 4mM L-Glutamine, 1mM sodium pyruvate, 500 µg/ml 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.). This was followed by incubation at room temperature for 20 minutes and addition of an extra 4.5ml serum-free medium. 293T cells were transfected with the transfection mix and incubated at 37°C in a humidified 5% CO₂ incubator. Viral supernatants were harvested 48hrs post transfection, followed by filtration through 0.45-µm PVDF syringe filter (Millipore) and 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 specifications.

**Western Blot Analysis of siRNA GFP Reporter**

Western blot analysis using anti-GFP antibody was performed on lysates from differentiated U937 cells which were transduced with the *piLenti-siRNA-GFP* phage for 24hrs and infection with *E. coli-4A* for 3 hrs followed by incubation for 0, 3, 6, 24, and 48hrs. The cells were washed, lysed and the lysate at each time-point was used for Western blot analysis using the GFP monoclonal antibody as previously described (Unlap and Jope, 1997b). Specifically, 5.2µg of each respective 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. After the 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 three hours. After 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 three times with PBS/0.1% Tween 20 and then washed an additional three times with 100 ml volumes of PBS/0.1% Tween 20 for 5 minutes. The membrane was probed with a 1:5000 dilution of horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Invitrogen) in 10 ml Blotto for 2hrs at 4°C. GFP protein was visualized using an ECL kit for the detection of HRP-labeled antibodies on Western blots (Fisher, Inc). The blot was placed in a film cassette and autoradiography was performed by exposing and developing the X-ray film (Hyperfilm-ECL, GE). The relative intensities of the protein bands were assessed using imageJ software from NIH and plotted as a function of time.

siRNA transduction, bacterial infection and bacterial isolation from macrophages

Recombinant E. coli-4A cultures were grown on Lennox L (LB) broth containing 50 µg/ml ampicillin and the expression of the recombinant protein is induced by 1mM IPTG as described in the bacterial culture conditions. U937 cells were differentiated for 48 hours in culture flasks at a cell density of 2.5 x 10^6 cells/flask. Seeded cells were transduced with piLenti-siRNA-GFP phage at an MOI of 5:1 for 24 hours, and then infected with E. coli-4A for 3 hours, followed by incubation for 0, 3, 6 and 24 hours. The cells were washed, lysed and the lysate was used for mRNA isolation and RTPCR.
analysis using methods as previously described (Unlap et al., 2000). For lysis, cells were incubated for 10 min in 250μl of lysis buffer (0.1% Triton X-100 in PBS, pH 7.4) and vortexed vigorously. The lysate was centrifuged at 13,000 x g for 5 mins to pellet the bacteria. The supernatant was aspirated and the pellet was washed three times with 250μL 1x PBS containing 1% Tween 80. The pellet was resuspended in 25μL SDW and then used for RNA or protein isolation.

**RNA isolation and purification**

The bacterial pellet from each time point was heat lysed at 94°C for 10 minutes and the lysate was centrifuged at 13,000 x g for 10 minutes at 4°C. The supernatant was transferred to fresh RNase free microcentrifuge tube and then incubated at 37°C with 2 U/μL DNase 1 in 1/9th volume of 10X DNase buffer (RiboPure™ Bacteria Kit – Life Technologies) for 30 minutes to digest the genomic DNA. The sample was then treated with a volume of DNase Inactivation Reagent equal to 20% of the volume of RNA treated to each sample for 2 minutes at room temperature with occasional mixing, followed by centrifugation at 13,000 x g for 1 minute at 4°C. The DNase free supernatant was collected and used for RTPCR.

**RTPCR experiments**

The mRNA samples were used along with two sets of primers designed to amplify cDNAs corresponding to *M. smegmatis* 1.4Kb *mce4A* (5’-GAGGAGCCATGGATGTCAAGCAGAAGGCACCGCCAA-3’ / 5’-GGAAGGAAGCTTGAAGTCGTCCCTTTCCCGA-3’) and 0.41Kb 16s *E.coli* ribosomal RNA gene (5’-CAACGAGCGCAACCCT-3’ / 5’-
GGTTACCTTGTTACGACTT-3`). RT-PCR was carried out by one-step RTPCR kit (Qiagen) according to the manufacturer’s instructions. Specifically, using a thermocycler, RTPCR was carried out at 50°C for 30 min, 95°C for 15 min, and 40 cycles at 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min., followed by a long extension time of 10 min at 72°C. Ten microliters of each PCR product were fractionated on a 1% Tris acetate-EDTA gel, and the relative intensities of the reverse-transcribed amplicons were assessed using imageJ software from NIH and plotted as a function of time.

**E. coli-4A macrophage invasion assay time-course following siRNA transduction**

U937 cells were differentiated with PMA and seeded at $2.5 \times 10^5$ cells per well in 12-well plates, followed by incubation for 24hrs. Seeded cells were either transduced or not-transduced for 24 hours with *pILenti-siRNA-GFP* construct at a MOI of 5:1. 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* were added to each well at a MOI of 10:1, followed by incubation at 37°C for 3 hours. Cells were washed 3 times with HyQ DMEM media containing 5% fetal bovine serum (FBS), 1% penn/strep and 100 μg/ml kanamycin to remove extracellular bacteria, followed by lysis after 0, 3, 6, 24, and 48hrs. For performing lysis, cells were incubated for 10 min in 500μl 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), followed by incubation at 37°C overnight. Recombinant *E. coli-4A* colonies were counted and the numbers of colonies that survived after 0, 3, 6, and 24hrs post infection was plotted versus time. Infection levels at 3,6, and 24hrs were compared to that of base line level (0hr) using Analysis of Variance (ANOVA).
Statistical Analysis

The effect of each treatment was assessed by comparing treated and non-treated samples using ANOVA. A p value $\leq 0.05$ was considered as significant.
RESULTS

Genomic DNA isolation and PCR amplification:

To clone each mce4 gene, DNA was isolated from log phase cultures of M. smegmatis and gene specific primers were used to amplify each of the mce4 genes to generate a 1.2 kilobase (Kb) (mce4A), 1.1Kb (mce4B), 1.0Kb (mce4C), 1.4Kb (mce4D) and 1.7Kb (mce4F) gene. Each PCR reaction was carried out at primer specific conditions in order to generate each fragment. An aliquot of each PCR reaction was resolved on 1% agarose gel against 1kb DNA marker. Our results (Fig. 1) showed that the use of these gene specific primers generated the correct fragment size for each gene, except for mce4F. The primers for mce4F failed to generate a band either in Ms.

Cloning into pTrcHis2-Topo:

Each of the mce4 operon genes was ligated into a prokaryotic expression vector, pTrcHis2-TOPO (Invitrogen, Carlsbad, CA). The generated plasmid contained an inducible promoter, P_{lac} that can be induced by isopropyl-β-D-thio-galactoside (IPTG).

Transformation and Quick screen for plasmid length & orientation:

Each of the mce4 operon genes was transformed into E. coli to generate corresponding E.coli-Ms4 clones: E.coli4A,E.coli4B,E.coli4C,andE.coli4D. The transformed E. coli clones were screened for correct plasmid size using a quick screen method (Unlap and Hu, 1995). Colonies expressing the correct size plasmid were used for plasmid isolation in order to determine the orientation using restriction digests. Orientation was also confirmed with gene specific forward primer and plasmid specific
reverse primer using PCR. The integrity of each clone was assessed by sequencing. *E. coli* expressing plasmids with the correct size and orientation were used for functional assays.

**Analysis of cloned mce4 gene products:**

To determine if the protein product of each gene was expressed in each *E. coli* clone, the recombinant proteins were isolated and used in Western blot analysis using 6xHis antibody. Our preliminary studies showed that the MCE4 recombinant proteins were synthesized and expressed in the *E. coli4A, E. coli4B, E. coli4C, and E. coli4D* (Fig. 2). However, we were unable to generate MCE protein product in *E. coli4F*.

**E. coli-mce4 Virulence Invasion Assays:**

After showing that each gene was transcribed and translated, an invasion assay was setup in order to determine whether or not the product of each gene conferred virulence to *E. coli* using MCF-7 human breast cancer cells. Our results showed that *E.coli4A, E.coli4B, E.coli4C, and E.coli4D* were able to infect MCF-7 cells. Of the four mce4 genes, mce4A demonstrated infectivity that appeared early and was sustained for the entire 72 hour period (Fig. 8a). Of the four Mtb-mce4 genes, Mtb-mce4A demonstrated the highest level of infection (Fig. 3).

**Cloning of mce4A siRNA in piLenti-GFP vector:**

A region of the mce4A gene in *M. smegmatis MC1552* was cloned into the piLenti-GFP vector to generate a siRNA against the mce4 mRNA. The cloning of this region of mce4A gene allowed the expression of a siRNA with two complementary 5’ and
3’ ends that will allow, after cellular processing, 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 facilitated the tracking of the constitutively expressed siRNA in vivo. The successful cloning of the fragment was then confirmed by digestion with EcoRV restriction enzyme.

Generation of piLenti-siRNA-GFP phage:

The phage was generated by transfecting piLenti-siRNA-GFP vector along with packaging plasmids into 293T cells using lentifectin® transfection agent (Applied Biological Materials, Inc.). Viral supernatants were harvested after 48hrs following transfection and filtered through 0.45-µm PVDF syringe filter, following by concentration using Ultra-Pure® lentivirus purification kit (Applied Biological Materials, Inc.). Viral titers were determined by RT-PCR using Lentiviral qPCR Titre Kit (Applied Biological Materials, Inc.) and the viral titer was found to be 10^6 IU/ml.

Western Blot Analysis

To confirm the expression of siRNA using the GFP reporter protein, Western blot analysis was performed on lysates from differentiated U937 cells which were transduced with the piLenti-siRNA-GFP phage for 24hrs, followed by infection with E. coli-4A for 3hrs and incubation for 0, 3, 6, and 24hrs. The cells were washed and lysed at each of the respective time-points and the lysate was used for Western blot analysis using a GFP monoclonal antibody. The relative intensities of the GFP protein bands were assessed using imageJ software and plotted as a function of time. Our results show that the
transfected cells constitutively expressed the GFP protein at all of the time points tested (Fig. 4).

**RTPCR**

To assess the effect of a designed mce4A siRNA molecular beacon construct on the mce4A mRNA levels, RTPCR analysis was performed on lysates from differentiated U937 cells which were transduced with the piLenti-siRNA-GFP phage for 24hrs, followed by infection with *E. coli-4A*, for 3hrs followed by incubation for 0, 3, 6, and 24hrs. The cells were washed and lysed and the intracellular bacteria were isolated at each time point of incubation. The bacterial sample from each time point was lysed and the mRNA isolated and purified using DNAse 1 enzyme treatment. The reverse transcripts were generated using RTPCR and the cDNAs were amplified using gene specific primers for mce4A and *E. coli* 16S rRNA gene as internal control. The degree of attenuation of *E. coli-4A* mce4A mRNA levels was compared between 3, 6, and 24hrs against that at 0hr and the results were found to be 0%, 81%, 40%, and 36%, respectively using *ImageJ* densitometry analysis software. Our results showed that mce4A siRNA attenuated *E. coli-4A* mce4A levels within infected macrophages as opposed to *E. coli* 16S rRNA internal control and the degree of attenuation of mce4A mRNA levels in *E. coli-4A* was found to be significant (*p* ≤ 0.05) (Fig. 5A & Fig. 5B).

**Invasion Assay:**

After confirming the expression of the GFP protein by Western blot analyses, invasion assay was carried out to determine the effect of the mce4A siRNA on macrophage infection. U937 macrophages were differentiated with 4nM PMA for 48
hours (Fig. 6), and were transduced with piLenti-siRNA-GFP phage for 24hrs followed by infection with E.coli-4A for 3 hours, and incubation for 0, 3, 6, and 24hrs. 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 (100µg/ml). The degree of attenuation of E. coli-4A infection was compared between 3, 6, and 24hrs against that at 0hr and was found to be 0%, 77%, and 60%, respectively. Our results showed that mce4A siRNA attenuated E.coli-4A infection in macrophages and the degree of attenuation of E. coli-4A infection was found to be significant (p ≤ 0.05) (Fig. 7).
Fig. 1. PCR amplification of *mce4A*, *mce4B*, *mce4C* and *mce4D* of *M. smegmatis*.

*Mce4* operon genes were PCR amplified from *M. smegmatis* MC2155 using gene specific primers and resolved on 1% agarose gel. The forward primer spanned the first 21 nucleotides from the beginning of the open reading frame and the reverse primer covered 21 nucleotides spanning the complementary strand to the 3’ end of the gene. The termination codon was omitted so that the product, MCE4A–D, will be expressed with a 6XHis tag and a myc tag.
Fig. 2. Expression of MCE4A-F recombinant proteins in *E. coli*4A-F. Immunoblot analysis was performed on cell lysates of stationary phase cultures of *E.coli*4A-F expressing the vector (V) or *mce*4A-F. Lysates were fractionated on SDS-PAGE and Western blotting was performed using mouse monoclonal anti-c-myc or anti-His primary antibody and goat antimouse HRP secondary antibody for localization of MCE4A-F. Molecular mass standards are indicated in kDa (Fisher).
Fig. 3. Ms-MCE4 proteins confer virulence to *E.coli*. *E.coli-mce4* clones were used to infect 2x10^6 MCF7 epithelial cells at an MOI of 10:1 for 2 hours. The level of infection was assessed by counting bacterial colony numbers at 24, 48 and 72 hours post-infection (n=3).
Fig. 4. GFP is immunodetected in transduced U937 cells infected with *E. coli-4A*. U937 macrophages were differentiated in 12-well plates for 48 hours with 4nM PMA at a cell density of 1 x 10^6 cells per well and transduced with *piLenti-siRNA-GFP* construct at a MOI of 5:1 for 24 hours. Transduced U937 cells were infected with IPTG-induced log phase cultures of recombinant *E. coli-4A* at a MOI of 10:1 for 3 hours, followed by extensive washing and incubation for 0, 3, 6, and 24. Total cell lysate was obtained at each time point and fractionated on SDS-PAGE, followed by Western blotting 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) was accomplished using the Immobilon Western Chemiluminescent HRP Substrate (ECL) System® (Millipore) and the relative intensities of the protein bands were assessed using ImageJ software from NIH and plotted as a function of time.
Fig. 5. RTPCR demonstrates that *Mce4A* siRNA attenuates *mce4A* mRNA in *E. coli-4A* infected U937 cells. Differentiated U937 cells were transduced at MOI of 5:1 with *piLenti-siRNA-GFP* lentivirus for 24 hours and were infected with IPTG-induced log phase cultures of recombinant *E. coli-4A* at a MOI of 10:1 for 3 hours, followed by extensive washing and incubation for 0, 3, 6, and 24 hours. Cells were washed three times in 1x PBS, lysed in 0.1% Triton-X 100 lysis buffer and the bacteria isolated, washed three times in 1x PBS containing 1% Tween 80 and heat lysed at 94°C for 10 minutes to release the total bacterial RNA. The RNA samples were purified with 10X DNase buffer and used for RTPCR with gene specific primers against 1.4Kb *mce4A* (Fig 5A left panel) and 0.41Kb *16S* rRNA (Fig 5A right panel). The relative intensity of each of the bands in the RTPCR gels of *mce4A* were quantified using *ImageJ* (NIH) software and the numbers at 3, 6, and 24 hours post infection are compared with those at 0hr using ANOVA. n = 3; P ≤ 0.05 (Fig 5B).
Fig. 6. U937 cells are differentiated into macrophages. U937 human histiocytic lymphoma cells are differentiated to mature macrophage cells using Phorbol-12-myristate-13-acetate (PMA) (40X). Differentiated U937 cells were chosen for the infection in order to mimic latent tuberculosis macrophage model.
Fig. 7. *Mce4A* siRNA attenuates *E.coli-4A* infection in U937 cells. Differentiated U937 cells were transduced at MOI of 5:1 with *piLenti-siRNA-GFP* lentivirus for 24 hours and were infected with IPTG-induced log phase cultures of recombinant *E. coli-4A* at a MOI of 10:1 for 3 hours, followed by extensive washing and incubation for 0, 3, 6, and 24 hours. Cells were washed extensively in 1x PBS, lysed in 0.1% Triton-X 100 lysis buffer and the lysate was plated on LB agar plates containing ampicillin (100µg/ml) followed by incubation at 37°C overnight. Colonies are counted and the numbers at 3, 6, and 24 post infection are compared with those at 0hr using ANOVA. n=4; *P ≤ 0.05*
DISCUSSION

This study was necessitated because of three reasons: (i) latent tuberculosis is a major problem for both developing as well as developed countries, (ii) there is no rapid and specific diagnostic test against latent TB, and (iii) there are no effective treatments against eradication of 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, especially mce4A, could be used for the attenuation of mce4A mRNA. This hypothesis was tested in differentiated U937 cells infected with recombinant E. coli expressing mce4A gene.

In order to design the molecular beacon siRNA construct, it was first necessary to determine which gene in the mce4 operon had the greatest degree of virulence. Our studies using recombinant E. coli expressing various mce4 operon genes (Fig 1 and Fig 2) have showed that mce4A gene conferred the greatest degree of virulence to the host E. coli (Fig 3). Based on these results, we designed a siRNA sequence with a stem loop structure, with the 29 nucleotide loop region homologous to a region of the mce4A mRNA and the 5 base pair stem regions at either ends complimentary to each other. The stem-loop structure facilitates efficient entry of the siRNA into the bacterium and upon hybridizing to the target mRNA, it will cause the degradation of the latter. The transduction of the mce4A siRNA into the U937 cells ensured its constitutive presence inside the macrophage (Fig. 4). This molecular beacon design tested the rationale that in the presence of the target mce4A mRNA, the loop region will hybridize with the target mRNA sequence, there by inducing the bacterial interference machinery to cleave the
siRNA-mRNA duplex and reducing the levels of intact \textit{mce4A} mRNA. The results show that the molecular beacon \textit{siRNA} construct attenuates its target mRNA levels in macrophages infected with \textit{E. coli-4A} (Fig. 5A & Fig. 5B). Since it has been shown that the mycobacterium utilizes the product of \textit{mce4A} for survival on cholesterol for carbon and energy source (Senaratne et al., 2008; Saini et al., 2008; Miner et al., 2009; Xu et al., 2007), the degradation of the \textit{mce4A} mRNA will lead to its reduced survival. Our study tested the ability of the \textit{mce4A} siRNA to attenuate recombinant \textit{E. coli-4A} infection in macrophages and the results show that the molecular beacon \textit{siRNA} construct significantly attenuated its target mRNA in infected macrophages as evidenced by significant reduction in \textit{E. coli-4A} infection in the host macrophages (fig. 7).

Thus, a molecular beacon can be designed against one of the \textit{mce4} operon genes in \textit{M. smegmatis} that facilitates the attenuation of mycobacterial \textit{mce4A} mRNA responsible for mediating latent infection in macrophages.
REFERENCES


Reference Text:


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alpha, iNOS, IL-6, and IL-12 in bovine alveolar macrophages. Mol Cell Biochem 302: (1-2) 1-7. Doi:10.1007/s11010-006-9395-0.
CONCLUSION

In summary, first we were able to determine the most infective gene of the mce4 operon of *M. smegmatis* and *M. tuberculosis*. This was done by cloning each of the individual mce4 operon genes, *mce4A, mce4B, mce4C, mce4D*, and *mce4F* into *E. coli*, expressing these genes in *E. coli*, followed by cell invasion assays. Based on these experiments, *mce4A* was determined to be the most infective and we were then able to design a siRNA molecular beacon against this most infective gene. We then showed that the *mce4A* siRNA molecular beacon was able to detect mycobacterial infection in macrophages while a random molecular beacon did not elicit any positive signals.

After successfully localizing mycobacterial infection in macrophages, we constitutively expressed the siRNA molecular beacon in U937 macrophages. This was achieved by cloning the siRNA construct into a lentiviral vector and transducing differentiated U937 cells overnight. The expression time-course of the siRNA was confirmed for over a period of 48 hours with a GFP reporter using fluorescence microscopy and western blots.

We tested the ability of the *mce4A* siRNA to attenuate *mce4A* mRNA levels in infected macrophages. This was done by infecting transduced U937 macrophage cells with *E. coli* expressing the *mce4A* gene, followed by incubation and lysis at various time points to isolate the intracellular bacteria, washing and lysing of the bacteria to isolate the RNA, purifying the RNA, generating cDNA from the RNA using RT-PCR with gene specific primers against *mce4A* and *E. coli* 16S internal control, and finally,
densitrometrically assessing the degree of attenuation of transcripts at various time points. Our results demonstrated that the mce4A siRNA was able to significantly knock down the mce4A mRNA levels in the intracellular bacteria.

Finally, we tested the ability of the siRNA molecular beacon construct to attenuate mycobacterial infection in macrophages. This was achieved by infecting siRNA transduced U937 macrophage cells with E. coli expressing the mce4A gene or M. smegmatis, incubation and lysis at various time points to isolate the intracellular bacteria, followed by plating and comparison of the results with that of the baseline control in order to assess the degree of attenuation of infectivity. Results from these experiments demonstrated that the mce4A siRNA was able to significantly attenuate both E. coli-4A and M. smegmatis infection in macrophages.

Thus in conclusion, we have demonstrated that a siRNA molecular beacon can be designed against the most infective gene of the mce4 operon, which can be used to successfully detect and attenuate mycobacterial infection in macrophages.