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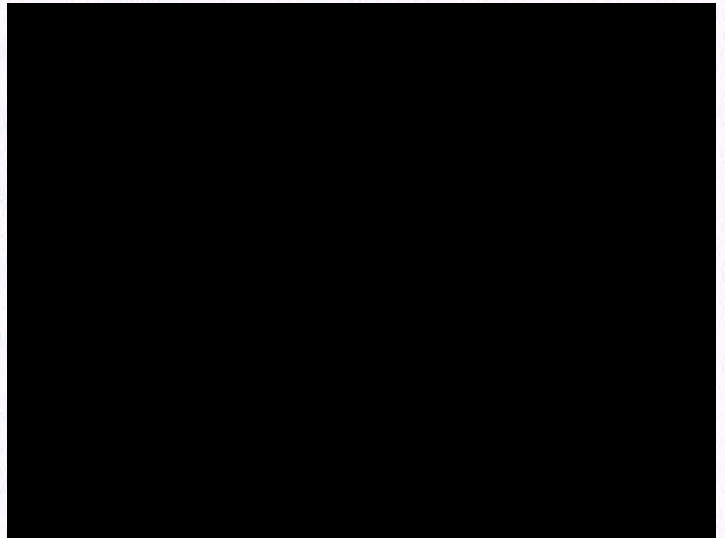
The University of Southern Mississippi

COMPARATIVE ANALYSIS OF ANTIVIRAL RESPONSES IN MOUSE

EMBRYONIC STEM CELLS AND IN MESENCHYMAL STEM CELLS

by

Jundi Wang

A Thesis
Submitted to the Graduate School
of The University of Southern Mississippi
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Approved:

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ABSTRACT

COMPARATIVE ANALYSIS OF ANTIVIRAL RESPONSES IN MOUSE

EMBRYONIC STEM CELLS AND IN MESENCHYMAL STEM CELLS

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Embryonic stem cells (ESCs) are cells that have unlimited capacity for self-renewal and differentiation. These properties make ESCs a great cell source for application in regenerative medicine. When used for cell therapy, ESC-derived cells could be placed in a wounded area that is likely to be exposed to various pathogens. However, it is not well-understood whether ESCs and ESC-derived cells have active antiviral responses against infectious agents from the environment. To answer this important question, I comparatively analyzed the antiviral responses of ESCs and mesenchymal stem cells (MSCs, C3H10T1/2 cell line) to infectious agents. Using the expression of type I interferon (IFN) as a benchmark of antiviral responses, our results indicated that the type I IFN were robustly induced in C3H10T1/2 cells, but not in ESCs, when they were exposed to polyinosinic:polycytidylic acid (poly(I:C), a dsRNA viral analog) and La Crosse Virus (LACV). Our results also showed that TLR3, RIGI and MDA5, the receptors for viral RNA, are expressed at lower levels in mouse ESCs (mESCs) than in C3H10T1/2 cells. However, mESCs are susceptible to LACV infection resulting in cell death, which can be reduced by IFNβ pretreatment. Furthermore, IFNβ induced expression of ISG15, PKR and dsRNA receptor genes that play key roles in antiviral responses. In conclusion, mESCs are deficient in type I IFN expression, but they have functional mechanisms that mediate the antiviral effects of type I IFN.

ACKNOWLEDGMENTS

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CHAPTER I

INTRODUCTION

Embryonic Stem Cell

Embryonic stem cells (ESCs) have been intensively studied for the past several

years due to their pluripotency and self-renewal capacity (Wobus & Boheler, 2005;

Keller, 2005). Pluripotency is a unique property of ESCs that allows them to differentiate

into any of the three germ layer cells. Therefore, ESCs are considered a promising cell

source for regenerative medicine. ESCs are derived from an early developmental stage of

the embryo called a blastocyst, which consists of outer trophoblast cells surrounding the

inner cell mass (ICM). The ICM is composed of undifferentiated cells with the ability to

form the three primary germ layers (endoderm, mesoderm and ectoderm), which will

further differentiate to all tissues and organs (Fig. 1).
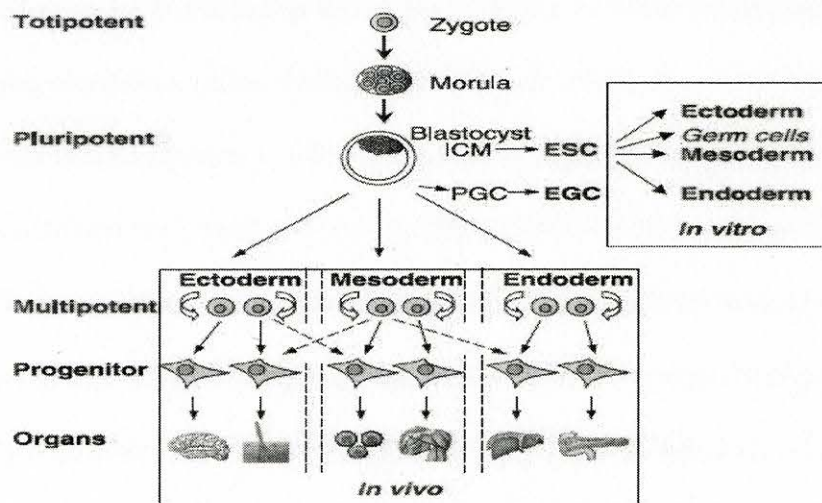
Figure 1. ESCs and Their Differentiation Potential (Wobus & Boheler, 2005)

Maintenance of pluripotency without further differentiation is necessary for the study of ESCs. Under in vitro culture conditions, ESCs require leukemia inhibitory factor (LIF) which prevents ESCs differentiation by binding to heterodimeric receptors and results in the activation of the JAK/STAT (Janus kinase-signal transducer and activator of transcription) pathway, which is essential and sufficient to maintain the pluripotency of ESCs (Niwa, 2007). Pluripotency refers to the capacity of ESC differentiating to different cell lineages (Wobus & Boheler, 2005; Keller, 2005). In the absence LIF, ESCs can differentiate into specialized cell types such as dendritic cells, cardiomyocytes, endothelial cells, neurons, smooth muscle cells and hepatocytes if proper conditions are provided (Ying, Stavridis, Griffiths, Li & Smith, 2003; Maltsev, Wobus, Rohwedel, Bader, & Hescheler, 1994; Risau et al, 1988; Lee, Lumelsky, Studer, Auerbach, & Mc 2000; Drab et al, 1997; Jones, Tosh, Wilson, Lindsay, & Forrester, 2002; Fairchild et al, 2000). Self-renewal is defined as the ability of ESCs to divide indefinitely while maintaining pluripotency (He, Nakada, & Morrison, 2009). It is well established that the self-renewal and pluripotency in ESCs are mainly regulated by a set of transcriptional factors, including Oct4, Sox2 and Nanog. The Oct4-Sox2-Nanog transcriptional regulatory network forms a positive feedback loop which allows these transcription factors to regulate each other by binding to anyone of their promoter regions, and negatively regulates the expression of differentiation promoting genes (Smith, 2001). Moreover, it has been reported that Oct4 and activated JAK/STAT3 pathway can cooperatively induce Kruppel-like factors such as klf 2, 4, and 5 to maintain ESC self-renewal (Pinney & Emerson, 1989). Therefore, Oct4, Nanog and Sox2 play the most important roles in maintain the properties of ESCs.

The self-renewal capacity and pluripotency give ESCs great potential in the field of regenerative medicine. Many studies have reported that ESCs-derived cells have the potential to treat human diseases, such as neurodegeneration, diabetes, and myocardial infarction (Svendsen & Smith, 1999; Soria et al, 2000; Klug, Soonpaa, Koh, & Field 1996). However, there are many obstacles still need to be overcome before ESCs are used as a source of cell based therapy. For example, it is known that self-renewal of undifferentiated ESCs can result in the development of teratomas if unpurified ESC-derived cells are transplanted into the patient (Keller, 2005). Furthermore, the issue of immunological rejection needs to be resolved, as it will cause the destruction of transplanted cells via activation of the recipient's immune system. Antiviral responses have been extensively studied in other types of cells, but whether or not ESCs and ESC-derived cells have any functional innate immunity has not been investigated. Since ESCs are normally residing in a sterile environment of the womb, they may not be exposed to pathogens from the outside environment during the early stage of development (Levy, 2007). However, for purposes of clinical application, ESCs must be differentiated and purified in vitro before being transplanted into patients. After transplantation, these cells will be potentially challenged by the infectious agents; therefore, the capacity of innate immunity is critical for the functionality and survival of implanted cells. As already suggested by previous studies, human ESCs (hESCs) and hESC-derived endothelial cells do not have mature immune function (Foldes et al., 2010; Chen, Yang, & Carmichael, 2010). The molecular basis of the innate immunity deficiency in ESCs has not yet been studied in details. The objective of this project is to investigate the antiviral responses as a critical part of innate immunity in mouse ESCs (mESCs).

Mesenchymal Stem Cells

Compared with ESCs, MSCs are more differentiated stem cells which can respond to different infectious agents. MSCs are adult stem cells with limited capacity of self-renewal and differentiation to closely related cell lineages (Williams & Hare, 2011). They were initially isolated from the bone marrow stroma, which have the potential to differentiate into mesoderm-derived cells, such as osteoblasts, chondrocytes, adipocytes, and myotubes (Williams & Hare, 2011; Minguell, Erices, & Conget, 2001). It is now known that MSCs exist in different tissues, such as umbilical cord blood, adipose tissue, and epithelial tissues (Ren et al., 2012; Lee et al., 2004; Jiang et al., 2002). Therefore, MSCs are also regarded as promising source of cell-based therapy for a wide range of degenerative diseases.

The C3H 10T1/2 cells were established in 1973 from 14- to 17-day old C3H mouse embryos. These cells display fibroblastic morphology in cell culture and are functionally similar to MSCs that can differentiate several cell types, such as adipocytes, pericytes/smooth muscle cells, and endothelial cells (Proweller, Pear, & Parmacek, 2005; Pinney & Emerson, 1989; Wang et al., 2010; Tang, Otto, & Lane, 2004). In this project, C3H 10T1/2 cells, a well-characterized MSC line that are highly responsive to different infectious agents (Wang et al., 2013), will be used for comparative analysis of the innate immunity in mESCs.

Antiviral Responses

Antiviral response is a critical component of innate immunity and plays very important roles against viral infections. Innate immunity and adaptive immunity are two components of the immune system. Innate immunity is the first line of an organism's defense against infectious agents. The innate immunity system is composed of mechanical, chemical and cellular elements. In mechanical element, the physical barrier, such as epidermis and mucosa, protects the organism from pathogens invasion. The chemical element is divided into three components: pathogen recognition, proteins or peptides-mediated microbe hydrolysis, and cytokines and chemokines that orchestrate the immune response. The third element is the cellular element, which includes epithelial cells, mast cells, dendritic cells, NK cells and phagocytic cells. These cells form a complicate antiviral network to protect organisms from infectious agents by different mechanisms (Basset, Holton, O'Mahony, & Roitt, 2003). In this project, we will focus on the study of antiviral responses in ESCs and MSCs.

It was well-established that innate immunity plays an important role in the pathogen-recognition and subsequent signaling transduction to protect cells from pathogenic attacks (Medzhitov & Janeway, 2000). Innate immunity can immediately respond to invading pathogens by recognizing conserved structures termed pathogen-associated molecular patterns (PAMPs) (Mogensen, 2009). During early innate immune activation, these PAMPs will be detected by pattern-recognition receptors (PRRs), proteins expressed on the cell surface or in the cytosol. After PRR activation, the downstream signaling pathways are triggered by activating a multitude of transcription factors, such as interferon regulatory factors (IRFs) and nuclear factor-κB (NF-κB),

which coordinately regulate the expression of type I interferon (IFN) and cytokines

(Akira, Uematsu, & Takeuchi, 2006). Through paracrine and autocrine signaling, IFNs

and cytokines participate in different aspects of innate immune as well as adaptive

immune responses (Mogensen, 2009).

The members of toll-like receptors (TLRs) family are the major class of PRRs and

are expressed on the cell surface or the membranes of endosomes in most cell types

(Miettinen, Sareneva, Julkunen, & Matikainen, 2001). TLRs are composed of an

extracellular ligand-binding domain, which recognizes and binds to the conserved

structures of pathogens, and a cytoplasmic Toll/interleukin-1 receptor homology (TIR)

domain that plays an important role in signal transduction (Carty & Bowie, 2010). After a

ligand binds to TLRs, adaptor molecules are recruited to the cytoplasmic domain of TLRs

and trigger downstream signal pathways (Mogensen, 2009). So far, 10 members of the

TLR family have been identified in mice (designated TLR1 through TLR10). TLR1, 2, 4,

5, 6, and 10 mainly detect bacterial components (Mogensen, 2009; Jin et al., 2007; Kim

et al., 2007). The rest of the TLRs (TLR3, 7, 8, and 9) mainly recognize nucleic acids

(Liu et al., 2008; Kawai & Akira, 2008).

The Retinoic Acid-Inducible Gene-I-like receptors (RLR) are another family of

PRRs. All RLR members are expressed in the cytoplasm. Retinoic Acid-Inducible Gene-I

(RIG-I), Melanoma Differentiation-Associated Gene 5 (MDA5), and Laboratory of

Genetics and Physiology 2 (LGP2) are the members of the RLR family. Although all of

these receptors can detect dsRNA, RIG-I preferentially detects short dsRNA and MDA5

mainly binds to long dsRNA (Kato et al, 2008; Pippig et al, 2009). The structure of RLRs

consists of a DExH/D (Asp-Glu-X-His/Asp)-box RNA helicase domain and a C-terminal

domain (Mogensen, 2009; Matsumiya & Stafforini, 2010). Two Caspase Activation and Recruitment Domains (CARDs) are located at the N-termini of RIG-I and MDA5, but LGP2 lacks a CARD domain (Yoneyama & Fujita, 2007). In the inactivated conformation, the C-terminal domain of RLR prevents CARDs from binding to the downstream adaptor molecules until RLR binds to viral dsRNA (Matsumiya & Stafforini, 2010; Yoneyama & Fujita, 2007; Saito et al., 2007). The binding of dsRNA to the C-terminal domains results in conformational change in C-terminal domain of RIG-I or MDA5. Then, the CARDs will be released and binds to the adaptor protein such as IFNβ promoter stimulator 1 (IPS-1) at the mitochondrial membrane to ultimately activate transcription factors that lead to the antiviral response (Matsumiya & Stafforini, 2010).

Double stranded RNA-activated protein kinase (PKR) is composed of two functional domains, an N-terminal dsRNA binding domain (dsRBD) and a C-terminal kinase domain containing the major phosphorylation site (Nallagatla, Toroney, & Bevilacqua, 2011). It plays important roles in antiviral response. The viral dsRNA is recognized by the dsRNA binding motifs (dsRBM) located at N-terminal dsRBD via minor groove interactions. The binding of viral dsRNA causes conformational changes in PKR and leads to autophosphorylation and dimerization. In addition to selectively activating the transcription of genes involved in the immune responses, PKR also causes a general inhibition of transcription, translation and host cell proliferation that limit viral replication (Mogensen, 2009). Table 1 lists the major microbial components and PRRs.

Table 1

Recognition of Microbial Components by PRRs

Receptor	Cellular Localization	PRR-Recognized Microbial Component(s)	Species of Microorganism(s)
TLRs			
TLR1/TLR2	Cell surface	Triacyl lipopeptides	Bacteria
TLR2/TLR6	Cell surface	Diacyl Lipopeptides	Mycoplasma
		Lipoteichoic acid	Gram-positive Bacteria
TLR2	Cell surface	Lipoprotein	Various pathogens
		Peptidoglycan	Gram-positive and negative bacteria
		Lipoarabinomannan	Mycobacteria
		Porins	Neisseria
		Envelope glycoproteins	Viruses
		GPI-mucin	Protozoa
		Phospholipomannan	Candida
		Zymosan	Fungi
		β-Glycan	Fungi
TLR3	Cell surface Endosomes	dsRNA	Viruses
TLR4	Cell surface	LPS	Gram-negative bacteria
		Envelope glycoproteins	Viruses
		Glycoinositolphospholipids	Protozoa
		Mannan	Candida
		HSP70	Host
TLR5	Cell surface	Flagellin	Flagellated bacteria
TLR7/8	Endosome	ssRNA	RNA viruses
TLR9	Endosome	CpG DNA	Viruses, bacteria, protozoa
RLRs			
RIG-I	Cytoplasm	dsRNA (short), 5'-triphosphate RNA	Viruses
MDA5	Cytoplasm	dsRNA (long)	Viruses
Miscellaneous			
PKR	Cytoplasm	dsRNA, 5'-triphosphate RNA	Viruses

After PRRs bind to their ligands, a number of different signaling pathways are activated depending on the binding of different sets of adaptor molecules. As shown in Figure. 2. In TLR-induced signaling pathways, ligand binding induces TLR dimerization and binding of adaptor molecules to the cytoplasmic TIR domain, such as Myeloid differentiation factor 88 (MyD88) or TIR-domain-containing adapter-inducing IFNβ (TRIF) following the binding of a ligand (Mogensen, 2009). MyD88 and TRIF are involved in regulating inflammatory genes and type I IFN expression. In the case of the signaling pathway mediated by TLR1, 2, 4, 5, 6, 7, 8, and 9, MyD88 binds to the TIR domain of the TLR and phosphorylates Interleukin-1 receptor-associated kinase (IRAK) and transforming growth factor-activated protein kinase 1 (TAK1), causing the activation of either the NF-κB or MAPK pathway (Mogensen, 2009; Burns et al., 1998). On the other hand, TRIF plays essential roles in TLR3 mediated antiviral pathway. The recognition of viral dsRNA by TLR3 recruits TRIF to the receptor. It was reported that the binding of TLR4 and bacterial component can also trigger TRIF-mediated downstream pathway. Then, TRIF binds to TNFR-associated factor 6 (TRAF6) and receptor-interacting protein 1 (RIP1) and ultimately activates the NF-κB and IRF3/7 pathway (Akira et al., 2006).

In the RLR mediated signaling pathway, dsRNA binding triggers signaling via CARD-CARD interaction between the receptor and the adaptor protein IPS-1 (Matsumiya & Stafforini, 2010). Then, the TNF receptor-associated death domain (TRADD) recruits to IPS-1 and forms IPS-1-TRADD complex (Michallet, 2008), which eventually phosphorylates IRF3 and IRF7 through the activation of TANK-binding

kinase 1 (TBK1) / I-κB kinase ε (IKKε) (Takahashi et al, 2006), which leads to the

expression of type I IFN and cytokines.
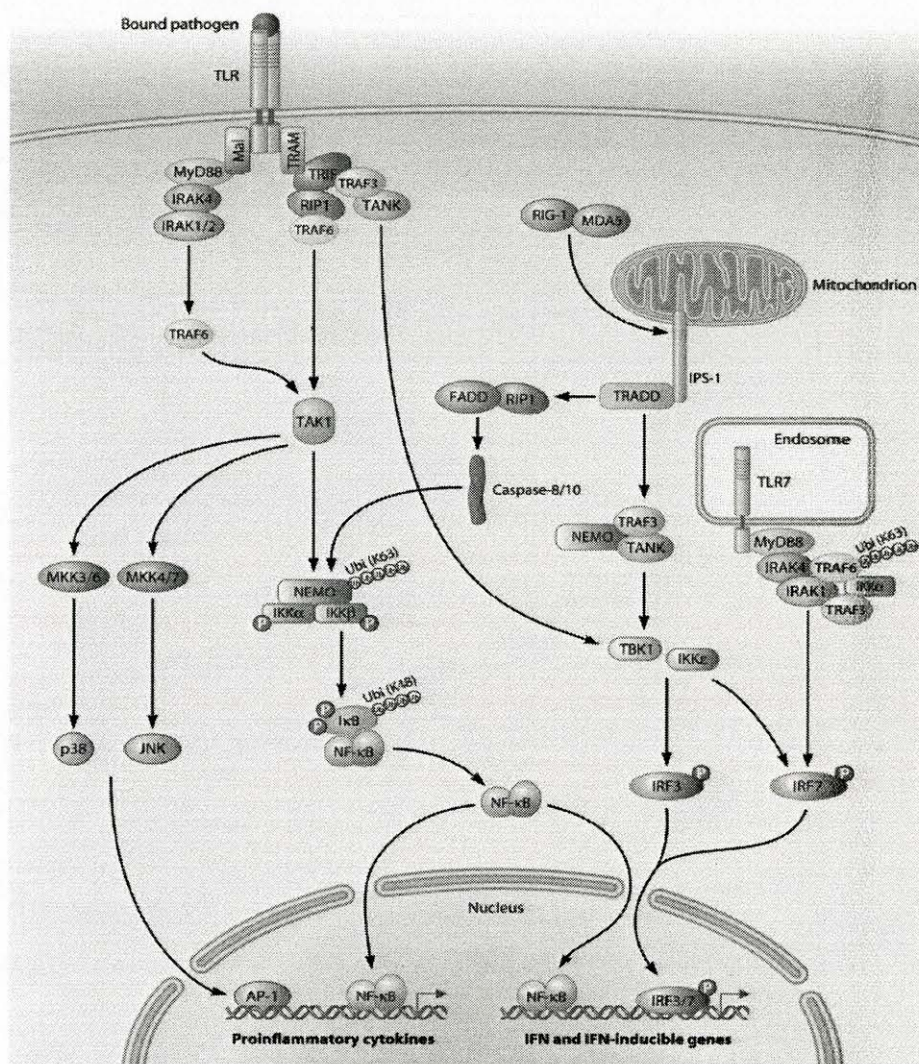
Figure 2. The Signaling Pathways Involved in Innate Immunity (Mogensen, 2009).

Type I IFN (IFNα and IFNβ) gene expression is mainly mediated by PRR

signaling pathways via activation of IRF3 and IRF7 (IRF3/7) (Akira et al., 2006;

Yoneyama et al., 1998). As shown in Figure 3, IFNα and IFNβ are secreted into the

surrounding tissue. Depending on the state of the cell, they affect different physiological

events via autocrine and paracrine signaling mechanisms. In normal cells, type I IFN

have been demonstrated to play important roles in inhibiting viral replication (Siren, Pirhonen, Julkunen, & Matikainen, 2005). On the other hand, type I IFN may cause the death of the infected cells as a mechanism of antiviral response (Takaoka & Yanai, 2006). Although type I IFN are best known for their antiviral activities, they also regulate several other cellular activities, such as cell differentiation (Boo & Yang, 2010; Clemens & McNurlan, 1985).

Figure 3. The Overview of the Signaling Network for Type I IFN in Innate Immunity (Takaoka & Yanai, 2006).

Signaling Pathway Activated by Type I Interferon

As mentioned above, the PRR-mediated antiviral pathways can activate NF-κB and IRF3/7, and induce expression of type I IFN. Once secreted, IFN bind the interferon-alpha/beta receptor (IFNAR) (Boo & Yang, 2010), which recruits and activates JAK and leading to STAT1 and STAT2 phosphorylation (De et al., 2001). The phosphorylated STAT1 and STAT2 translocate into the 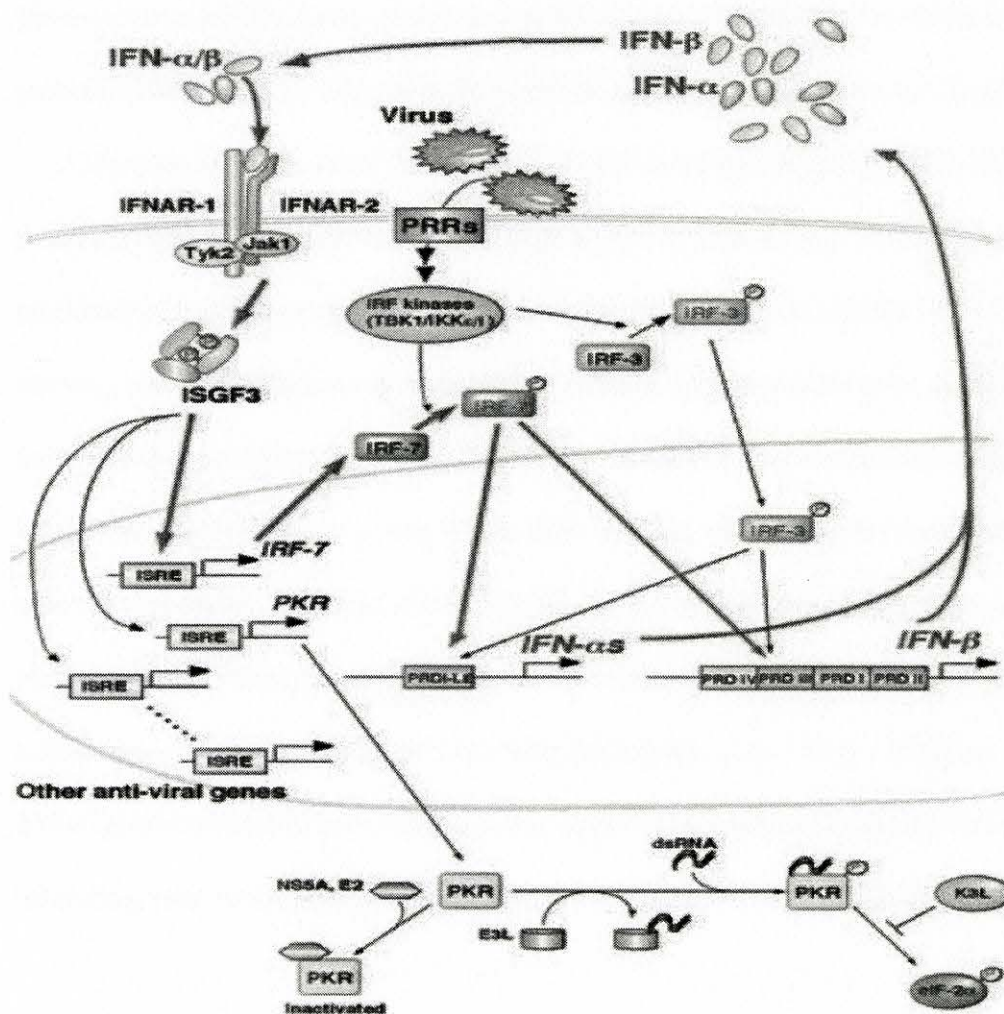nucleus and induced the expression of numerous genes, known as interferon-stimulated genes (ISGs). The best studied ISGs include Mx proteins, PKR, and 2',5'oligoadenylate, which play key roles in antiviral responses.

Mx proteins are a small family of GTPases. A unique property of Mx GTPases is their antiviral activity against a wide range of RNA viruses, such as Influenza and some members of the Bunyavirus family (Haller, Staeheli, & Kochs, 2007). GTP-binding and carboxy-terminal effector functions of Mx protein play important roles in recognizing viral nucleocapsid-like structures. Eventually, these viral nucleocapsid proteins are trapped and sorted into locations where they become unavailable for the generation of new virus particles (Haller & Kochs, 2002). As previously mentioned, activated PKR phosphorylates eukaryotic initiation factor 2 alpha (eIF2α) and blocks viral gene translation via protein synthesis inhibition (Nallagatla et al., 2011). Whereas, 2',5'oligoadenylate activates RNase L that causes degradation of viral RNA thereby inhibiting viral replication (Boo & Yang, 2010; Li, Blackford, & Hassel, 1998).

CHAPTER II

OBJECTIVE AND SIGNIFICANCE

ESCs have attracted enormous attention in recent years with the expectation that they could be used as a source for cell-based therapy. While the benefit of research on ESCs in medical applications is exciting, currently there is limited understanding of the basic physiology of ESCs and their derived cells. When used for cell therapy, ESC-derived cells would be placed in a wounded area that is likely to be exposed to various pathogens. Therefore, their fate and functionality may depend on their innate antiviral responses to deal with a hostile environment. Innate immunity as the first line of defense has been intensively investigated in a wide variety of somatic cells. It is generally believed that most cell types, if not all, have acquired innate immunity. However, recent studies indicated that ESCs do not respond to a wide range of infectious agents including bacterial LPS and dsRNA (Foldes et al., 2010; Chen et al, 2010). Our recent study demonstrated mESCs are unable to express type I IFN when exposed to viral infections, indicating that they do not have functional antiviral mechanisms (Wang et al., 2013). Surprisingly, we recently found that ESCs are able to respond to IFNβ and express the genes that confer antiviral activities. However, the molecular mechanisms involved have not been elucidated. The proposed study aims to understand the antiviral responses of ESCs and how they are affected by the antiviral effect of IFN. This study may open up an important area in ESC research for understanding the development of antiviral mechanisms during embryogenesis and how the immunogenic signals affects ESC physiology. The findings from this study could provide valuable information to prepare ESC-derived cells for their application in regenerative medicine.

CHAPTER III

METHODS

Cell Culture

mESCs (D3 cell line) were obtained from ATCC. They are used for the majority of the experiments in this study and maintained in Dulbecco's modification of Eagles's medium (DMEM) (Mediatech, Inc. , Manassas, VA) containing 15% fetal bovine serum (FBS) (ATLANTA biological, Lawrenceville, GA) with leukemia inhibitory factor (LIF) (EMD Millipore Corporation, Billerica, MA) and 100 unit/mL penicillin and 100 µg/mL streptomycin. They were routinely maintained in cell culture dishes coated with 0.1% gelatin. C3H10T1/2 cells were cultured in minimum essential medium (MEM) (Mediatech, Inc., Manassas, VA) containing 10% FBS and antibiotics at the concentrations mentioned above. All cells were maintained at 37 °C in a humidified incubator with 5% CO_2.

Cell Treatment

D3 and C3H10T1/2 cells were plated at ~40% and ~70% confluence, respectively, and cultured for 24 h before the experiments. Poly(I:C) (Sigma-Aldrich, St. Louis, MO) was transfected into the cells with DharmaFECT reagent (Thermo Scientific, West Palm Beach, FL). For poly(I:C) transfection experiments, control cells were transfected with DharmaFECT reagent only. For viral infection, LACV (a gift from Dr. Fengwei Bai, The University of Southern Mississippi) viral stocks were added to the cell culture at the concentrations as specified in individual experiments. The culture medium and treated cells were collected at different time periods and used for various analyses.

Spectrophotometric Analysis of Cell Viability

Cell viability was determined by colony size and by cell number after toluidine blue (TB) staining. The cells treated with poly(I:C) were fixed with methanol for 15 minutes. The cells collected from the live virus infection experiments were treated with 2% paraformaldehyde (PFA) first for virus inactivation. After PFA treatment, the cells were similarly fixed with methanol for 15 minutes. The cells were stained with TB for 30 minutes after phosphate buffered saline (PBS) (Thermo Scientific, West Palm Beach, FL) washing. After 30 min incubation, the cells were washed with water and air-dried, followed by using 2% sodium dodecyl sulfate (SDS) to extract the TB. OD_{630} value was measured by a Bio-Tek Instruments ELX800 microplate reader and analyzed with KC junior software (Bio-Tek Instruments, Inc, Winooski, VT).

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted using Tri-reagent (Sigma-Aldrich, St. Louis, MO). cDNA was prepared by M-MLV reverse transcriptase (Promega, Madison, WI). RT-qPCR was performed using SYBR green ready mix (Bio-Rad, Hercules, CA) on a MX3000PTM RT-PCR system (Stratagene, La Jolla, CA). The mRNA level from RT-qPCR was calculated using the $\Delta\Delta Ct$ method (Pfaffl, 2001). β-actin mRNA was used as a calibrator for the calculation of relative mRNA of the tested genes. Sequences of gene-specific primers are listed in Table 2.

Table 2

Sequences of Gene-specific Primers

Gene	Forward Primer	Reverse Primer
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IFNβ	CCCTATGGAGATGACGGAGA	ACCCAGTGCTGGAGAAATTG
IFNα	CTGCTGGCTGTGAGGACATA	AGGAAGAGAGGGCTCTCCAG
RIG-I	ATTCAGGAAGAGCCAGAGTGTC	GTCTTCAATGATGTGCTGCAC
MDA5	CGATCCGAATGATTGATGCA	AGTTGGTCATTGCAACTGCT
TLR3	CTTGCGTTGCGAAGTGAAGAA	CCAATTGTCTGGAAACACCCC
TLR4	TGCACTGAGCTTTAGTGGTTGC	GACCCATGAAATTGGCACTCAT
Oct4	AGTTGGCGTGGAGACTTTGC	CAGGGCTTTCATGTCCTGG
Nanog	TTGCTTACAAGGGTCTGCTACT	ACTGGTAGAAGAATCAGGGTC
Sox2	GACAGCTACGCGCACATGA	GGTGCATCGGTTGCATCTG
PKR	AAGCAGGAGGCAAGAAACG	TGACAATCCACCTTGTTTTCGT
ISG15	AGGTCTTTCTGACGCAGACTG	GGGGCTTTAGGCCATACTCC
IFNAR	GACAACTACACCCTAAAGTGGAG	GCTCTGACACGAAACTGTGTTTT

Protein Analysis by Flow Cytometry

To determine the protein level by fluorescence labeling, treated cells were released by 0.25% Trypsin-EDTA (GIBCO, Grand Island, NY) and fixed with 80% ethanol for 30 minutes at 4°C, then washed with PBS that contains 2% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) for 20 minutes. Cells were incubated with primary antibodies (ICAM1) (Santa Cruz Biotechnology, Inc., Santa Cruze, CA) at 1:100 dilution in PBS containing 2% BSA for 2 h at room temperature. The cells were then incubated with secondary antibodies conjugated with FITC (1:100) (Santa Cruz

Biotechnology, Inc., Santa Cruze, CA) in PBS with 2% BSA for 1 h at room temperature. The cell suspension was examined through an Accuri C6 flow cytometer. The fluorescence intensity, which correlates with the protein level, was determined with CFlow software (BD Accuri Cytometers, Inc., Ann Arbor, MI).

IFNβ and Cytokine Assay

The culture medium collected from treated cells was used to determine secreted IFNβ and cytokines. IFNβ was quantified with an Enzyme-lined Immune Sorbent Assay (ELISA) kit (PBL interferon source, Piscataway, NJ) that detects mouse IFNβ according to the manufacturer's instruction. IL6 was analyzed with a Luminex cytokine assay kit (EMD Millipore Corporation, Billerica, MA) and determined with a MAGPIX instrument (EMD Millipore Corporation, Billerica, MA). The data was analyzed according to the method described by Parbhakar et al. (Prabhakar, Eirikis, & Davis, 2002).

Plaque Assay

Titers of LACV in culture medium collected from infected cells were determined in vero cells by plaque assay as described by Bai et al (Bai et al., 2005). Briefly, 95-100% confluent vero cells were infected with 10000x dilution of culture medium collected from infected cells and incubated at 37°C in 5% CO_2 incubator for 1h. After virus adsorption, medium was removed completely and cells were overlaid with DMEM-agarose and further incubated for 4 days. The plaques were counted after staining with second overlay containing 4% (vol/vol) neutral red.

CHAPTER IV

RESULTS

In order to determine the antiviral responses in mESCs, we treated the cells with two commonly used two agents. Poly(I:C) is a synthetic double-stranded RNA used as a viral analog. In many publications, poly(I:C) has been shown to activate antiviral responses via binding to RIG-I, MDA5, TLR3, and PKR (Heim, 2005; Hu et al., 2011; Zimmer, 2011). It has also been reported that poly(I:C) can cause cell cycle interruption via PKR-induced eIF2α phosphorylation, which results in protein synthesis inhibition (Garcia, Meurs, & Esteban, 2007). LACV belongs to Bunyaviridae family, which can cause encephalitis. The LACV genome is composed of three single-stranded, negative-sense RNA segments. Although poly(I:C) is commonly used to mimic a viral infection, it is a synthetic compound which may not reflect antiviral response of ESCs under the physiological conditions. Therefore, LACV, a live virus, is chosen for this study since it is known to cause lytic cell death of mammalian cells (Verbruggen et al., 2011). It has been shown that LACV can activate PKR and induce eIF2α phosphorylation in fibroblasts (Streitenfeld et al., 2003). Additionally, the non-structured protein of LACV can also induce mitochondrial cytochrome C release and Caspase activation in cell-free extracts and promote neuronal apoptosis and mortality in a mouse model (Colon-Ramos et al., 2003).

Double Stranded RNA (dsRNA) Transfected to Mouse ESCs (mESCs) Inhibits Cell

Proliferation

We transfected poly(I:C) into mESCs since this is a commonly approach that

induce antiviral responses in many cell types. The most notable effect of transfected

poly(I:C) on mESCs at the cellular level was the inhibition of cell proliferation. As

shown in Fig. 4A, the colonies of poly (I:C)-transfected cells were much smaller than the

control colonies as determined by microscopic analysis (Fig. 4A), correlating with

markedly reduced cell numbers determined by toluidine blue cell staining (Fig. 4B, left

panel). The proliferation inhibition effect of transfected poly(I:C) was also found in

C3H10T1/2 cells (Fig. 4B, right panel).
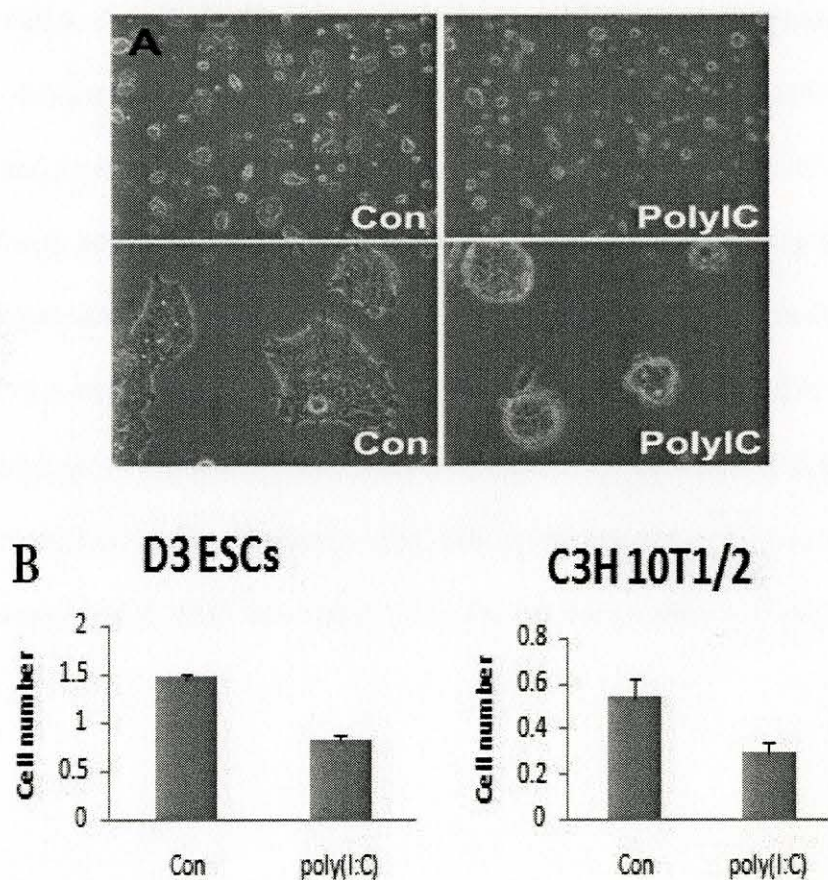
Figure 4. Effects of poly(I:C) on mESC colony formation and cell proliferation. A and B) Cells were transfected with 300 ng/mL poly(I:C). Control (Con) represents cells transfected with DharmaFECT without poly(I:C). A) After incubation for 40h, the colonies were examined under a phase contrast microscope and photographed with a digital camera (100x, upper panels; 400x, lower panels). B) Cell proliferation was measured by cell number indirectly determined from toluidine blue staining (absorbance at 630 nm). The values are means ± SD of an experiment performed in biological triplicate (B).

mESCs Fail to Express and Produce IFNβ, IL-6, and ICAM1 in Response to dsRNA

We analyzed the mRNA levels of IFNβ, intercellular adhesion molecule 1 (ICAM1), and proinflammatory cytokine gene such as Interleukin-6 (IL-6) in the cells transfected with poly(I:C). The mRNA levels of IL-6 and IFNβ were negligible in mESCs compared with C3H10T1/2 cells (Fig. 5A and B). When examined at the protein level by ELISA and Luminex assay, neither IFNβ nor IL-6 was detected in the medium collected from poly(I:C)-transfected mESCs, whereas they were readily detectable in the samples from C3H10T1/2 cell cultures (Fig. 5D). Similarly, poly(I:C) can significantly induce expression of ICAM1 in C3H10T1/2 cells, but not in mESCs (Fig. 5C).
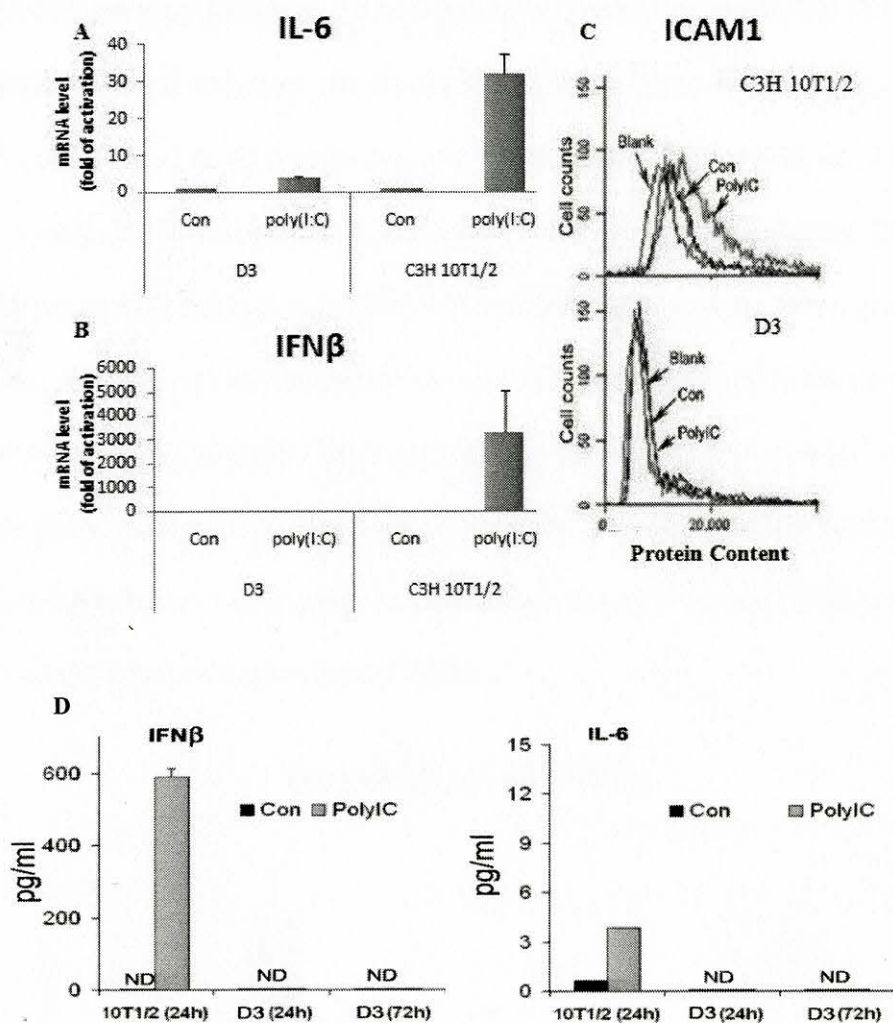
Figure 5. mESCs fail to express and produce IFNβ, IL-6, and ICAM1 in response to dsRNA. A-D, D3 mESCs and C3H10T1/2 cells were treated with 300 ng/mL poly(I:C). A and B), mRNA levels of tested genes were analyzed by RT-qPCR. The results are expressed as fold-activation where the mRNA level in control cells is designated as 1. The results are means ± SD of three independent experiments. C) Expression profiles of ICAM1 proteins in D3 mESCs and C3H10T1/2 cells were determined by flow cytometry. The representative experiments in all panels were repeated at least twice with similar results. D) The culture medium collected at different time points was analyzed for IFNβ by ELISA (detectable range 15.6-1000 pg/mL) and IL-6 by Luminex assay (detectable range 0.64-10000 pg/mL). ND: not detected. The values for IFNβ are means ± SD of a representative experiment performed in triplicate. The values for IL-6 are average of a representative experiment performed in duplicate.

Relative Expression Levels of dsRNA Receptors in mESCs and C3H10T1/2 Cells

Innate antiviral responses are mediated by PRRs. These receptors induce the

expression of IL-6 and IFNβ by activating the transcription factors IRFs and NF-κB

(Carty & Bowie, 2010; Matsumiya & Stafforini, 2010; Yoneyama & Fujita, 2007).

Therefore, we analyzed the basal mRNA levels of PRR genes. Among the genes we

tested, RIGI, MDA5 are viral dsRNA receptors which are expressed in the cytosol. TLR3

is another viral dsRNA receptor, but it can express both on cell surface and in cytosol.

We observed that they are much lower in mESCs than in C3H10T1/2 cells (Fig. 6). This

observation indicated that the negligible gene expression of IL-6 and IFNβ in mESCs

may be related to the low expression of PRRs.

Basal level of PRRs
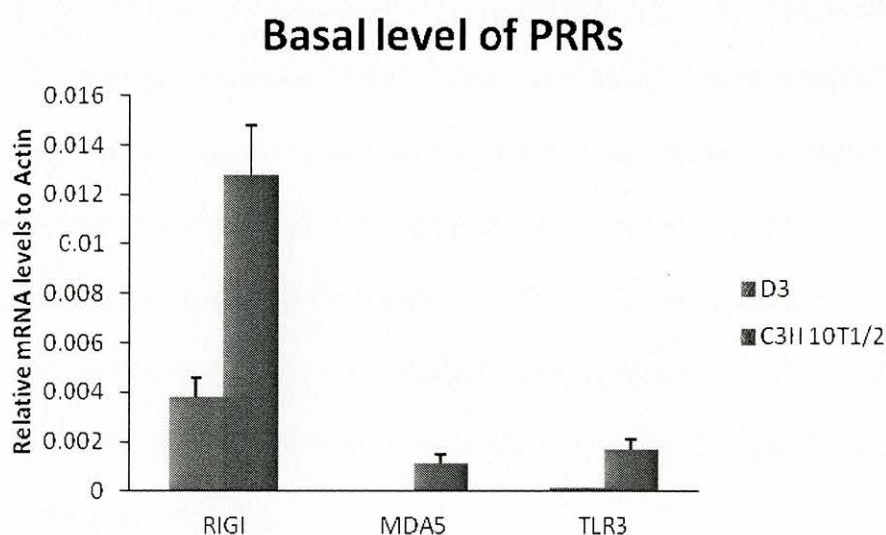
Figure 6. The expression levels of RNA receptor genes in mESCs are much lower than in C3H10T1/2 cells. Cells were plated at 30-40% confluence and cultured for 24h without treatment. The mRNA levels of tested genes were analyzed by RT-qPCR. The results are means ± SD of three independent experiments.

mESCs Are Susceptible to The Cytopathic Effects of LACV Infection

While poly(I:C) has been used as a dsRNA viral analog (Offermann et al., 1995), it is an synthesized RNA that does not have biological activities associated with viral infection. To investigate the responsiveness of mESCs to live viral infection, we used LACV, a negative sense, single stranded RNA virus, to determine whether mESCs can respond to live viruses. Because the proliferation of mESCs is much faster than C3H10T1/2 cells, mltiplicity of infection (M.O.I) of 10 and 1 were used in mESCs and C3H10T1/2, respectively, in LACV infection experiments. The cell number was then measured by spectrophototmetry where optical density is proportional to cell number. Our results demonstrated that the LACV challenge reduced the cell viability of mESCs and C3H10T1/2 cells (Fig. 7A and B), which was due to LACV-induced cytopathic effect (Fig. 7C). The viral infection of mESCs was confirmed by the expression of a LACV gene that encodes an M-segment protein (Gc protein) (Soldan, Hollidge, Wagner, Weber, & Gonzalez-Scarano, 2010). The infected cells were immunostained with monoclonal antibodies against the Gc protein (a gift from Dr. Samantha Soldan, University of Pennsylvania School of Medicine) followed by flow cytometry analysis. As shown in Fig. 7D, the expression of Gc protein was detected at 30 h and was significantly increased at 40 h post infection.
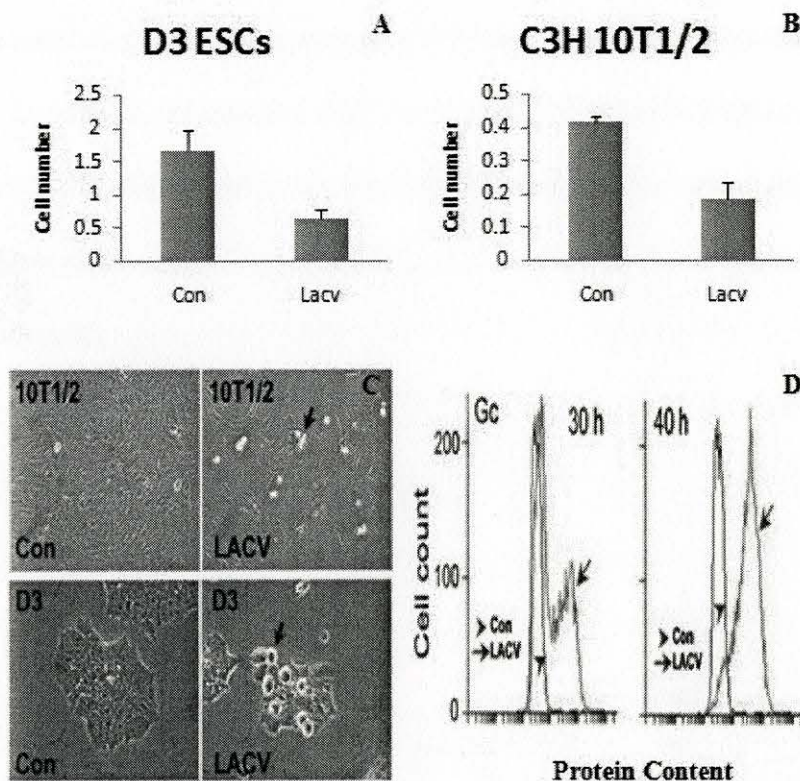
Figure 7. mESCs are susceptible to the cytopathic effects of LACV infection. A and B) D3 mESCs and C3H10T1/2 cells were infected with LACV at M.O.I=10 and M.O.I=1, respectively, for 48h. Viable cells were determined at 48 h post infection by toluidine blue staining. The values for D3 mESCs are means ± SD of three independent experiments, while the value for C3H10T1/2 cells is representative experiment performed three times. C) D3 mESCs and C3H10T1/2 cells were infected with LACV (M.O.I=1). The cells were examined under a phase contrast microscope and photographed (400 x) at 48 h for C3H10T1/2 cells and 60 h for D3 cells. Arrows denote detaching dead cells. D) Detection of LACV Gc protein in D3 cells infected with LACV (MOI=10) by flow cytometry. All experiments were performed at least twice with similar results.

mESCs Are Deficient in Expressing Type I IFN in Response to Viral Infection

In order to address the question of whether or not a live virus can induce antiviral responses in mESCs, we infected mESCs and C3H10T1/2 cells with LACV and analyzed the mRNA levels of antiviral genes. Even at very high M.O.I. (10), LACV only induced the negligible expression of type I IFNs in mESCs, but the induction in C3H10T1/2 cells is drastic (Fig. 8).
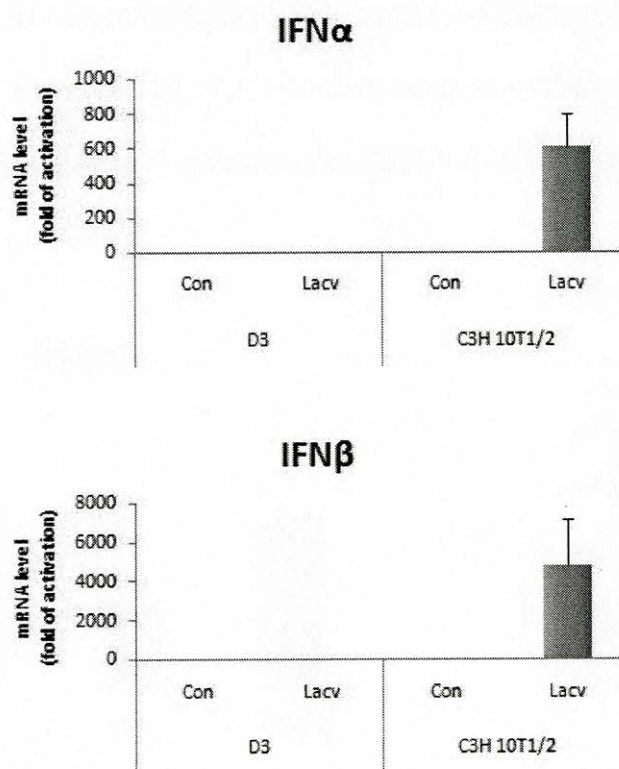
Figure 8. mESCs are deficient in expressing type I IFN in response to viral infection. D3 mESCs and C3H10T1/2 cells were infected with LACV at M.O.I=10 and M.O.I.=1, respectively. The mRNA levels of the tested genes were determined by RT-qPCR 24 h post-infection. The results are means ± SD of three independent experiments.

IFNβ Inhibits LACV Replication and Protects mESCs from LACV-Induced Cytopathic

Effects

It is clear that the mESCs are deficient in IFN expression in response to viral

infection and dsRNA. Since the expression of IFN and responsiveness to IFN are through

different signaling pathways (as illustrated in Fig.2 and Fig.3). It would be interesting to

see if mESCs can respond to IFN. We pretreated ESCs with IFNβ followed by LACV

infection. As shown in Fig. 9A, IFNβ pretreatment protected mESCs from LACV

induced cell death as judged by the increased number of viable cells after infection (Fig.

9A). Furthermore, the result of LACV viral titer analysis indicated that IFNβ treatment

can significantly inhibit LACV replication in mESCs as judged by the reduced viral titer

(Fig. 9B).
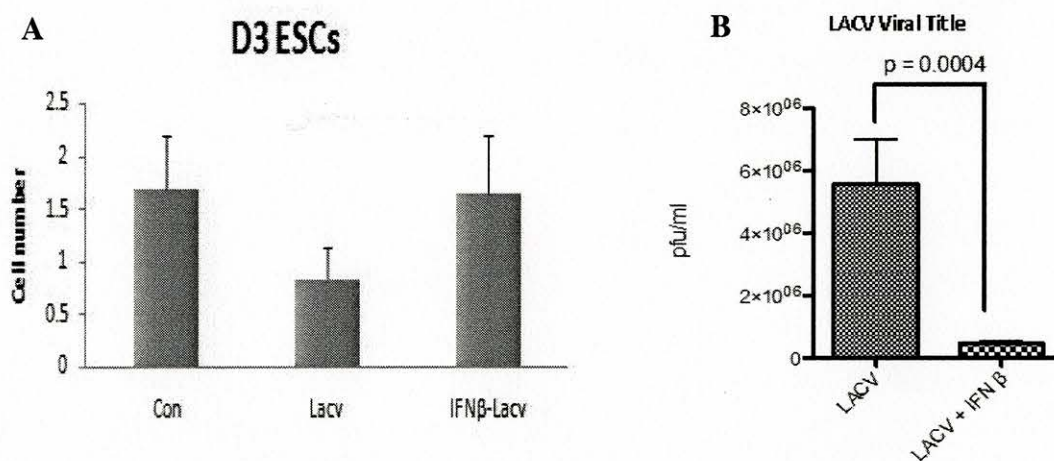
Figure 9. IFNβ can protect mESCs from LACV-induced lytic cell death. A) D3 mESCs were pre-treated with IFNβ (5000U/mL) for 24h. Then, the pretreated cells were infected with LACV at M.O.I=10 for 48h. Viable cells were determined at 48 h post infection by toluidine blue staining. B) The culture medium collected at 48 h post infection was analyzed for viral titer by plaque assay. The values are means ± SD of three independent experiments.

IFNβ Induces the Expression of dsRNA Receptors and Interferon-Stimulated Genes in

mESCs

To determine IFNβ-induced cellular responses, we analyzed the expression level of type I IFN receptors (IFNAR1) and genes that are known to be regulated by IFNβ. As indicated in the Fig. 10A, IFNAR1 was readily detected at the mRNA level in mESC although it is lower than in C3H10T1/2 cells. The mRNA levels of viral RNA receptors, including RIG-I, MDA5 and TLR3, were increased by IFNβ treatment (Fig. 10B). Finally, we analyzed the expression of IFNβ-stimulated gene 15 (ISG15), which was upregulated about 80 fold. Together, the results from these experiments confirm that mESCs are responsive to IFNβ and that the signaling pathways that mediate the effects of IFNβ are functional.
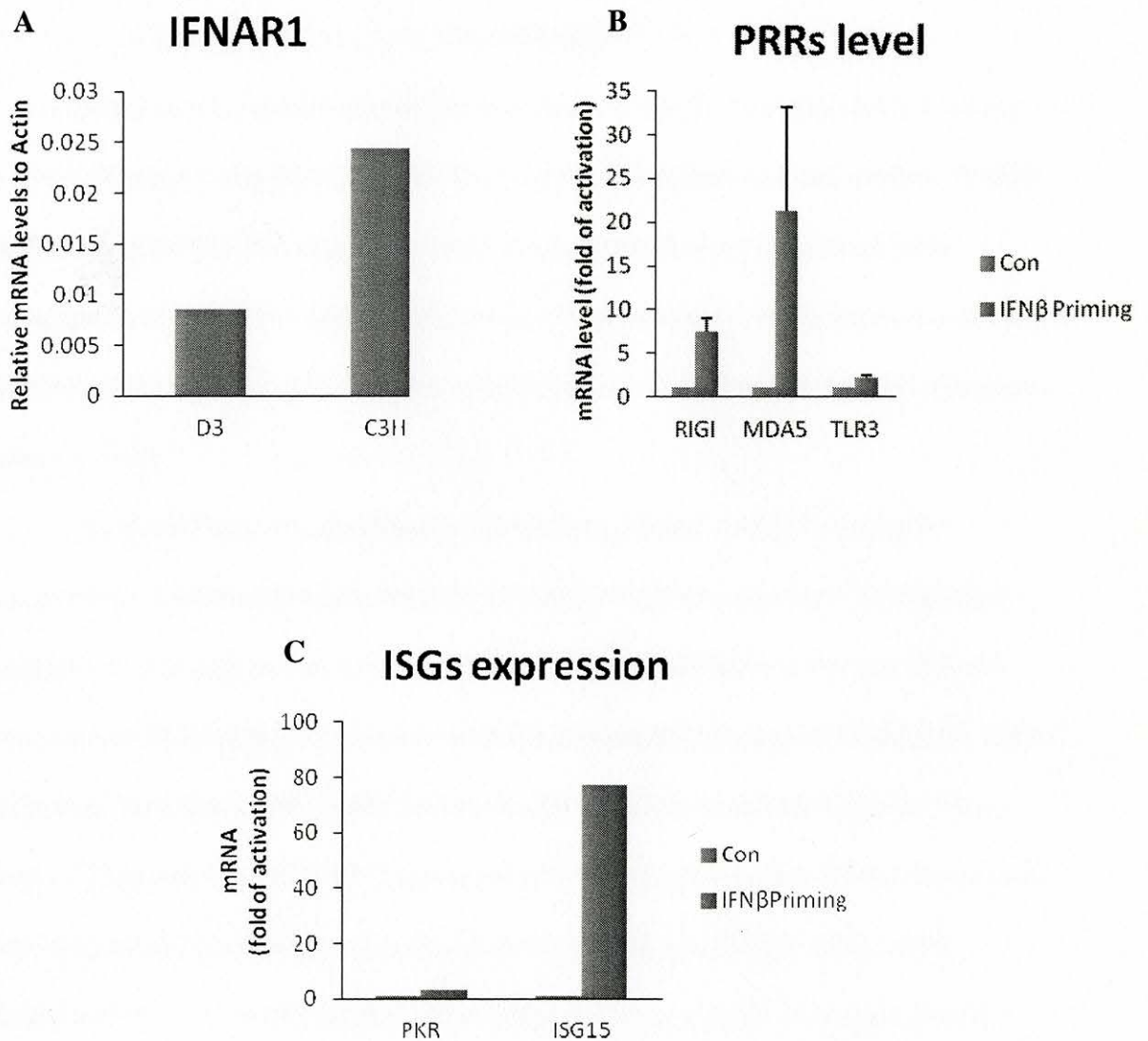
Figure 10. IFNβ induces the expression of dsRNA receptors and ISGs in mESCs. A) D3 mESCs were plated at 30-40% confluence and cultured for 24 h without treatment. B and C) D3 mESCs were treated with IFNβ (5000 U/mL) for 24 h. The mRNA levels of tested genes were analyzed by RT-qPCR. The results of PRRs levels are means ± SD of three independent experiments. The values of IFNAR1 and ISGs come from a representative experiment.

CHAPTER V

DISCUSSION

In this study, we investigated the responses of mESC to a viral dsRNA analog and to live La Crosse Virus (LACV) infection. While both stimuli induced a robust IFNα/β expression in C3H10T1/2 cells, they only induced very limited or no detectable transcription of IFNα/β in mESCs. These results suggest that mESCs are deficient in type I IFN expression, a central component of antiviral mechanisms in most types of somatic cells.

We have found that the defective IFNα/β expression in mESCs could be explained by the low expression level of TLR3 although we could not rule out other mechanisms that may be involved. In some cell types, poly(I:C) can induce IFN via activation of TLR3 at the cell surface or in the endosomes (Stetson & Medzhitov, 2006; Meylan & Tschopp, 2006; Nasirudeen et al., 2011). When transfected into the cells, poly(I:C) can induce robust IFN expression and other responses that are similar to those evoked by viral infection via cytoplasmic dsRNA receptors (Fortier et al., 2004; Matsumoto & Seya, 2008). mESCs were unresponsive to poly(I:C) that was directly added to the medium, likely due to the very low expression level of TLR3. However, poly(I:C) transfected into the mESCs showed a profound inhibitory effect on proliferation, a known effect of polyIC on many types of differentiated cells, indicating that the cytoplasmic receptors for poly(I:C) are active in mESCs. While we have provided substantial amount of evidence that poly(I:C) induced PKR activation is responsible for cell inhibition of ESCs, it is clear that the mechanisms mediating type I IFN is not functional in mESCs (Wang et al., 2013).

It is generally believed that MDA5 and RIG-I play primary roles in mediating viral RNA induced IFNα/β expression in the cytoplasm (Stetson & Medzhitov, 2006; Nasirudeen et al., 2011), while PKR also contributes to and modulates this process (Garcia, Meurs, & Esteban, 2007; Samuel, 2001). Since MDA5 is expressed at negligible level in mESCs, it is conceivable that PKR and/or RIG-I may mediate the effects of transfected poly(I:C). However, the RIG-I signaling pathway seems to be inactive since silencing RIG-I did not affect the effects of polyIC and that 3p-ssRNA (5'-triphosphate single- stranded RNA), the best studied ligands of RIG-I (Pichlmair, et al., 2006; Yoneyama & Fujita, 2007), failed to induce IFN in mESCs (unpublished data).

It was reported that LACV has developed certain mechanism to avoid the host innate antiviral responses. Repressing IFNα/β induction in host cells is one of such mechanisms (Haller, Kochs, & Weber, 2006). However, the capacity of LACV to repress IFNα/β induction seems to depend on the types of host cells, as we demonstrated in C3H10T1/2 cells in which LACV can induce strong transcription of IFNβ, but not in mESCs. The similar results were also found in dsRNA viral analog, West Nile Virus (WNV), and Sendai Virus (SeV) treated C3H10T1/2 and mESCs (data not shown). It seems that the failure to express IFNα/β in mESCs could be an intrinsic property of mESCs, even though they are sensitive to the cytopathic effect of LACV infection. This conclusion is in line with similar observations, recently reported by Wash et al (Wash, Calabressi, Franz, Griffiths, & Goulding, 2012), that herpes simplex virus (HSV) and influenza virus (a negative sense, ssRNA virus) caused cytopathic effects without evoking type I IFN in mESCs.

The antiviral responses include two major types of cellular responses: pathogen recognition/IFN production and IFN positive feedback regulation (Takaoka & Yanai, 2006). The best known function of type I IFN is their antiviral responses. In differentiated cells, IFN can enhance the antiviral responses of the cells by induction of several genes, known as IFN-stimulated genes (ISGs), which participate in various stages of antiviral responses. It is intriguing to find that mESCs do not have functional mechanism to synthesis IFN, yet they can respond IFN as demonstrated by the expression of the type I IFN receptor, expression of ISGs, and IFNβ-priming induced protection mESCs from LACV-induced cytopathic effects. These observations support a conclusion that mESCs have functional mechanisms to detect and respond to IFNβ although the cellular and molecular mechanisms involved remain to be investigated.

In summary, our study demonstrates that expression of type I IFN, a crucial part of antiviral responses, is underdeveloped in mESCs, but the IFNβ receiving and responding mechanisms are functional. This study may open up an important area in ESC research for understanding the development of antiviral mechanisms during embryogenesis and how the immunogenic signals affects ESC physiology.

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