Activation of NF-κB Transcription Factor During In Vitro Differentiation of Mouse Embryonic Stem Cells

Natalya A. Ortolano

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Activation of NF-κB Transcription Factor During In Vitro Differentiation of Mouse Embryonic Stem Cells

by

Natalya A. Ortolano

A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of the Requirements for the Degree of
Bachelor of Science
in the Department of Biological Sciences

May 2015
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Honors College
ABSTRACT

Embryonic stem cells (ESCs) are a promising cell source for regenerative medicine. However, recent studies indicated that ESCs and ESC-derived cells (ESC-DCs) lack functional innate immunity against various pathogens and inflammatory cytokines. This presents a barrier to clinical application, as ESC-DCs would be placed in a wound site and exposed to pathogens and inflammatory cytokines. Using mouse ESCs (mESCs) as a model, we recently demonstrated that they are deficient in expressing type I interferons (IFN) and inflammatory cytokines. To determine the molecular basis for this finding, this study examined the activation state of nuclear factor-κB (NF-κB), a transcription factor that plays a key role in mediating the antiviral and inflammatory responses. Our results indicated that NF-κB in mESCs was not activated by TNFα, a cytokine that is known to induce strong inflammatory responses in differentiated cells. However, NF-κB was activated when mESCs were differentiated to fibroblasts (ESC-FBs). This was confirmed by NF-κB translocation from the cytoplasm to the nucleus upon TNFα treatment. Once in the nucleus, NF-κB activates transcription of immune related genes, but the expression of its regulated genes was much lower than in naturally differentiated FBs. Further analysis by RT-qPCR revealed that the expression of some NF-κB pathway components is upregulated in ESC-FBs. We conclude that the lack of innate immunity in mESCs is, at least partly, due to the inactivated status of NF-κB in mESCs.

Key Words: NF-κB, innate immunity, mESCs, differentiation, regenerative medicine
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<th>Description</th>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>C3H</td>
<td>C3H10T1/2 Cell Line, Naturally Differentiated Fibroblasts</td>
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<td>D3-FBs</td>
<td>Fibroblasts Derived From D3 Cells</td>
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<td>DBA-FBs</td>
<td>Fibroblasts Derived From DBA Cells</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>ESC</td>
<td>Embryonic Stem Cell</td>
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<td>ESC-DC</td>
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<td>Fetal Bovine Serum</td>
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<td>hESC</td>
<td>Human Embryonic Stem Cell</td>
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<td>IkB</td>
<td>Inhibitor of Nuclear Factor-κB</td>
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<td>ICAM-1</td>
<td>Intracellular Adhesion Molecule-1</td>
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<td>ICM</td>
<td>Inner Cell Mass</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>IKK</td>
<td>Inhibitor of Nuclear Factor-κB Kinase</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>iPSC</td>
<td>Induced Pluripotent Stem Cell</td>
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<tr>
<td>LIF</td>
<td>Leukemia Inhibitory Factor</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>mESC</td>
<td>Mouse Embryonic Stem Cell</td>
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<td>MEF</td>
<td>Mouse Embryonic Fibroblast</td>
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<td>NF-κB</td>
<td>Nuclear Factor-κB</td>
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<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PRR</td>
<td>Pattern Recognition Receptor</td>
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<td>RT-qPCR</td>
<td>Real Time quantitative Polymerase Chain Reaction</td>
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<td>TBST</td>
<td>Tris-buffered Saline with Tween-20</td>
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<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
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<td>Tumor Necrosis Factor-α</td>
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<td>TNFR1/2</td>
<td>Tumor Necrosis Factor Receptor 1/2</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular Cellular Adhesion Molecule-1</td>
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INTRODUCTION

Stem Cells

Background

Stem cells are characterized by their capacity of self-renewal and cell potency. Self-renewal is defined as the ability of stem cells to proliferate while maintaining their undifferentiated state. Cell potency is the ability of a cell to differentiate into more mature cell types. There are two general categories of stem cells: embryonic stem cells (ESCs) and adult stem cells. An ESC is pluripotent meaning it can differentiate into any cell type of the adult organism. ESCs are important in early development of an organism. Adult stem cells, however, play a role in tissue repair throughout an organism’s life and have limited differentiation potential. Adult stem cells can be either multipotent or unipotent. A multipotent cell can differentiate into several similar cell types, while a unipotent cell can only differentiate into one cell type (Li & Xie, 2005).

Since this research is focused on ESCs, the following introduction is a brief discussion of ESCs and their roles in organism development, in vitro derivation, and basic characterization. ESCs are derived from the inner cell mass (ICM) of blastocysts. As shown in Figure 1, the isolation of ESCs from the ICM can be better understood with a basic explanation of mouse embryogenesis. First, a zygote is formed by the fertilization of an egg by a sperm. A zygote is totipotent, meaning that it has the potential to give rise to all the cells of the embryo as well as extra-embryonic tissues like the placenta. Further cell divisions and differentiation yield a blastocyst, composed of an outer layer of
differentiated cells known as trophoblasts and an ICM of undifferentiated, pluripotent cells (Wobus & Boheler, 2005). These cells are able to develop into any cell type derived from the embryo proper. When these cells are harvested and cultured in vitro, they are termed ESCs (Niwa, 2007).

**Figure 1:** An overview of mouse embryogenesis and ESC derivation (image from Wobus & Boheler, 2005).

ESCs, due to their pluripotent nature, have the ability to differentiate into cells of any of the three primary germ layers: ectoderm, mesoderm, and endoderm. Once a cell differentiates into tissue cells of one of the primary germ layers, they become multipotent (only able to differentiate into cells of that particular tissue type). The inner germ layer, the endoderm, has the potential to develop cells such as lung cells or liver cells. The middle germ layer, the mesoderm, has the potential to develop into cells such as red blood cells, muscle cells, and fibroblasts. The outer germ layer, the ectoderm, has the potential to develop cells such as epithelial cells and neurons (Wobus & Boheler, 2005).
As embryonic development progresses, organ development becomes necessary to form a functional organism. This is the point at which multipotent and unipotent adult stem cells form. Once the embryo is fully developed and exits the uterus, these adult stem cells reside in “stem cell niches” where they can repair damaged tissue as needed throughout the organism’s life. For example, hematopoietic stem cells reside in the bone marrow where they can be induced to differentiate into various types of blood cells as needed (Li & Xie, 2005).

**Characteristics of ESCs**

The most defining characteristic of ESCs is their ability to maintain their undifferentiated pluripotent state during in vitro propagation. The ability of ESCs to maintain pluripotency requires the careful maintenance of signals the cell receives. The cell must keep a perfect balance of promoting proliferation while down regulating differentiation. This delicate process is mediated by many transcription factors. The main transcription factors regulating this process are Oct3/4, Sox2, Nanog, and Stat3. Oct3/4 prevents differentiation of ESCs. Sox2 shares a similar role, often interacting with Oct3/4. Stat3 is the transcription factor activated by the JAK/STAT (Janus kinase signal transducer and activator of transcription) pathway promoting pluripotency in cells. Nanog seems to repress factors promoting differentiation, thereby promoting pluripotency and self-renewal. Specific expression levels of each of these transcription factors are vital for maintaining pluripotency through self-renewal. For example, overexpression of Oct3/4 and inhibition of Stat3 can cause differentiation (Niwa, 2007).

There are also significant differences between the cell cycles of ESCs and somatic cells. The mechanism of cell cycle regulation varies between the two. The G1 phase is
much shorter in ESCs than in somatic cells, and most ESCs are found in the S phase. As these cells differentiate, their cell cycle becomes more like somatic cells (White & Dalton, 2005).

ESCs also have a distinct morphology, as shown in Figure 2. ESCs have a distinctly round shape and a large nucleus-to-cytoplasm ratio. ESCs also have a tendency to form tightly packed colonies in vitro.

**Figure 2:** Morphology of a mESC colony (stained with toluidine blue)

*Maintenance of ESC pluripotency and Differentiation*

The first pluripotent cell lines were derived from teratocarcinomas, a germ cell tumor that occurs in the testes of mice and humans. It was discovered that these malignant tumors were not only composed of germ cells, but also embryonal carcinoma stem cells (Wobus & Boheler, 2005). In 1970, Kahan and Ephrussi created an embryonal carcinoma stem cell line by isolating these cells from a teratocarcinoma and propagating them in vitro. These cells were able to maintain their undifferentiated state in vitro for about 119 generations and were able to be differentiated into neural and mesenchymal cell types in vitro (Kahan & Ephrussi, 1970). While embryonal carcinoma stem cell lines made a good model for the study of embryonic cells, isolation of ESCs themselves from mouse embryos would allow for their direct study. It was not until 1981 that isolation of
ESCs from the ICM of mouse blastocysts was achieved and mouse ESCs (mESCs) were cultured in vitro. This led to the creation of the first mESC cell lines (Evans & Kaufman, 1981; Martin, 1981). Finally, in 1998 human ESC (hESC) cell lines were created following the isolation of inner mass cells of human blastocysts from in vitro fertilized human embryos (Thomson et al. 1998).

To create an ESC culture, inner mass cells are first isolated from a blastocyst, which forms about 4-5 days after fertilization. These are plated in culture medium containing bovine serum on a plate with mouse embryonic fibroblasts feeder layers. The feeder layer supplies factors necessary to maintain the pluripotent state of the ESCs (Amit et al., 2005). A greater understanding of the molecular mechanisms maintaining pluripotency in mESCs has permitted the growth of mESC cultures without the use of a feeder layer. The cytokine leukemia inhibitory factor (LIF) is commonly used in mESC cultures to inhibit differentiation and maintain a stem cell state. This cytokine activates JAK/STAT leading to the activation of Stat3, promoting proliferation and pluripotency while inhibiting differentiation (Niwa, 2007).

Manipulation of transcription factors can also be used to induce in vitro differentiation. When LIF is removed from mESC culture, Stat3 is inhibited, and differentiation occurs. Other chemicals such as retinoic acid can be added to culture to promote differentiation to different cell types as illustrated in Figure 3 (Niwa, 2007).
Promises of ESCs in Biomedical Applications and in Basic Research

It has been shown that hESCs can be differentiated into cardiomyocytes, neurons, endothelial cells, and many more cell types, showing great promise for organ replacement via cell therapy (Grinnemo et al., 2008). However, there are no current therapies utilizing hESCs due to many issues involving the ethical concerns and technical obstacles to their clinical application. For example, there is great concern about exposure to viruses by the mouse feeder layer and accumulation of genetic mutations in hESC cell lines (Hyun, 2010). However, the potential of use of hESCs in regenerative medicine has been supported by numerous mESC studies (Wobus & Boheler, 2005).
In order to utilize ESC derived cells (ESC-DCs) for therapeutic application, the ESC-DCs must fully integrate and function appropriately once transplanted to the targeted site of the organism. mESC derived cardiomyocytes, neural progenitor cells, and insulin producing cells have all been successfully transplanted into mice integrating fully into the tissue, functioning just as their in vivo counterparts. These transplants have successfully treated diseases such as Parkinson’s disease and diabetes in mouse models. Neural progenitor cells derived from hESCs have been successfully integrated into mice’s brains, showing specific functions once transplanted (Wobus & Boheler, 2005).

Most of the current knowledge available about stem cells is derived from mESC research. Some of these findings have been confirmed in hESCs. For example, Oct4, Sox2, and Nanog play important roles in maintaining pluripotency in both cell types (Schnerch et al., 2010). However, research has also found great differences between the two. For example, the use of LIF is not enough to maintain the stem cell state in hESCs, as it is in mESCs, indicating some differences in the mechanisms involved in maintaining pluripotency and differentiation potential. It has even been suggested that the LIF/STAT3 pathway in hESCs isn’t functional (Humphrey et al., 2004). Due to these differences, some of the research conducted using mESCs may not be applicable to hESCs (Scherch et al., 2010). For example, although Parkinson’s has been successfully treated in mouse models from both mESC and hESC derived neural progenitor cells, a clinical study using hESC derived neural cells to treat human patients had some negative long term effects and did not seem to improve the patient’s condition despite the success of the transplant itself (Freed et al., 2001). Further research should be conducted to
determine the long-term functionality of hESC-DC transplants in human subjects (Wobus & Boheler, 2005).

In addition to their potential for clinical application, ESCs make a great model for the study of early tissue development. This type of research can contribute to a better understanding of miscarriages, infertility, and even birth defects (Wobus & Boheler, 2005; Yu & Thomson, 2008). The studies of the function of certain genes in development are often conducted by inducing a mutation within a gene suspected to be involved with these defects. For example, the discovery of the role of Nanog in maintaining the pluripotent state involved its targeted mutation on homologous chromosomes (Wobus & Boheler, 2005).

**Obstacles to Biomedical Application of ESCs**

Although the characteristics of ESCs prove them to be ideal candidates for tissue engineering and regenerative medicine, many issues must be addressed before their clinical application. For example, undifferentiated ESCs form tumors known as teratomas in transplant patients. This is both a mark of their pluripotency and creates a risk of carcinogenic tumor development (Hentze et al., 2009). There is also the issue of obtaining a pure homogeneous population of the cell of interest. Additionally, there is the risk of immunorejection of both ESCs and their derivatives following transplantation due to mismatched major histocompatibility complexes between patient and donor (Drukker, 2004).

There is also concern about the genetic instability of ESCs over long periods of time in culture. Mutations occur naturally in cells, although they occur at lower rates in ESCs. The accumulation of these mutations over hundreds of cell divisions could lead to
mutations in important regulatory genes or even lead to the formation of carcinogenic cells. Also, the culture conditions of ESC cultures can affect the epigenetic modifications of the cells. For example, serum withdrawal can induce epigenetic modifications that can negatively affect cell potency and development (Wobus & Boheler, 2005).

Recent data indicates that both ESCs and their derivatives have an underdeveloped innate immune response, posing a potential problem when transplanted into the patient where the cells will be challenged by pathogens in the environment (Wang et al., 2013; Zampetaki et al., 2006; Rajan et al., 2008). Without a competent innate immune response, these transplanted cells would be defenseless against an attack from a pathogen, potentially putting the transplant at risk. This thesis research focuses on this deficiency.

**Social and ethical concerns in hESC research and alternatives to hESCs**

In addition to the technical issues, there are also ethical concerns involved in stem cell research. There has been great debate over the rights of human embryos and the ethics of using them as a source of hESCs. While many members of the public seem to believe that hESCs are obtained from aborted fetuses, this is not the case. hESCs are derived from excess embryos produced by in vitro fertilization for couples undergoing fertility treatment. Hundreds of eggs are fertilized in a petri dish during this process to form blastocysts. Since only a few of the blastocysts are implanted into the woman’s uterus, the rest are generally discarded. However, couples are given the option to donate these excess embryos to research. It is these in vitro fertilized embryos destined for destruction that are used to isolate hESCs and form hESC cell lines (Lo & Parham, 2009; Hyun, 2010).
However, many still feel there is an ethical issue with the destruction of embryos formed from in vitro fertilization. These people hold the view that even prior to implantation, embryos possess the same rights as any living individual and destruction of these embryos for any reason is murder. This belief likely stems from the misunderstanding that all embryos have the ability to form fully developed organisms, however, many of these in vitro developed embryos are unlikely capable of this if not further utilized. Those in support of ESC research argue that these embryos would be otherwise destroyed making their potential to form human life irrelevant since they would never be implanted in a uterus (Hyun, 2010).

In 2001, the Bush administration passed legislation supporting the belief that embryos deserve the rights granted to living individuals due to their potential for life. This legislation limited the creation of new hESC stem cell lines by permitting federal funding only to hESC research that utilized hESC stem lines that were already in existence. This presented many problems considering the possible exposure of these cell lines to zoonotic viruses from the mouse feeder layers they were grown on and the increasing amount of genetic mutations. These issues mandated that new hESC lines be created from excess embryos from in vitro fertilization (Hyun, 2010).

Due to this limitation during the Bush administration, many researchers sought to find alternatives to the use of hESCs. For example, Meissner and Jaenisch (2006) developed the altered nuclear transfer technique in which they created mouse embryos with a knockout for a gene responsible for implantation. These embryos possessed the ability to produce ESCs, but did not possess the potential to develop a fully formed, living organism since they were unable to implant onto a uterus or form a trophectoderm.
However, these cells would still require the use of hESCs as a comparative control before they could be used as a substitute (Meissner & Jaenisch, 2006; Hyun, 2010).

The development of induced pluripotent stem cells (iPSCs) offers the most appealing possible alternative to hESCs. iPSCs are differentiated cells that have been “reprogrammed” to revert back to a pluripotent ESC state. Takahashi and his group converted mouse fibroblasts to pluripotent stem cells using retroviruses to force the expression of transcription factors important in maintenance of the stem cell state (Oct 3/4, Sox2, Klf4, and c-myc) in the fibroblasts (Takahashi, 2006). This experiment was repeated and successful in human somatic cells, forming hiPSCs. While it appears these cells function like hESCs, meaning they are able to differentiate into cells of all three primary germ layers, there is still concern about the clinical use of these cells. Genetic differences between iPSCs and hESCs have been detected among other concerns. Until it is determined these cells are equivalent to hESCs, the use of them and their derivatives in cell-mediated therapy is questionable and requires systematic investigation (Takahashi et al., 2007).

**Innate Immunity**

*The Innate Immune Response*

To understand the implications of the lack of a developed innate immune response in ESCs and their derivatives, a brief introduction to the innate immune response is needed. Innate immunity plays a vital role as a cell’s first line of defense against microbial attack. The innate immune system consists of mechanisms to detect invading
pathogens and activate intracellular signaling pathways that lead to induction of immune genes to protect the infected cell, fight the pathogen, warn neighboring cells, and activate the adaptive immune system (Akira et al., 2006).

Cells are able to distinguish between self and non-self molecules via an array of pattern recognition receptors (PRRs) at the cell surface, in endosomes, or in the cytosol. Different PRRs bind to broad classes of molecular motifs that are typical of microbes (termed pathogen associated molecular patterns, or PAMPs). PAMPs are generally structural components of a pathogen that are necessary for function and are therefore constitutively expressed by the microbe with little alteration. A common example of a PAMP would be lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacterium, or double stranded RNA, which is commonly generated during viral infection. PAMPs are recognized by PRRs, leading to an innate immune response. PRRs are constitutively expressed in cells involved in innate immunity such as antigen presenting cells (dendritic cells and macrophages), B-cells, and structural tissue cells such as endothelial cells and fibroblasts. Once these PRRs recognize a PAMP, they induce a signaling cascade, which eventually converges at the point of transcription factor activation. These transcription factors induce the transcription of immune genes (Akira et al., 2006).

The immune genes are mostly those encoding cytokines and interferons (IFNs). Cytokines have a proinflammatory function in the cell, most often characterized as responding to bacterial infection. Common examples would be interleukin-6 (IL-6) and tumor necrosis factor α (TNFα). Cytokines promote vasodilation, recruiting more immune cells to the site of infection (Akira et al., 2006). Type I IFNs (IFNα/β) are more
specific toward antiviral responses and directly inhibit viral replication (Huang et al., 2006). For example, toll-like receptor 3 (TLR3) is a PRR located either on the membrane of a cell, a phagosome, or an endosome. It recognizes the double stranded RNA pattern found in viruses followed by recruitment of adaptor proteins, forming a signaling scaffold that transduces a signal to the point of activating nuclear factors such as nuclear factor-κB (NF-κB) and IRF-3. These translocate to the nucleus and bind to the IFNβ promoter, upregulating its transcription. IFNβ is then able to mediate cellular responses itself by interactions with cellular receptors on other infected cells and uninfected cells. Through signaling via the JAK-STAT pathway, it is able to upregulate the transcription of protein kinase R (PKR) and 2’-5’-oligoadenylate synthetase (2’5′OAS). Once these enzymes have been activated by interaction with double stranded RNA, they are able to induce cell-mediated effects. PKR phosphorylates eukaryotic initiation factor 2, inhibiting eukaryotic transcription. This prevents the cell from transcribing viral DNA. 2’5′OAS activates RNase L, which degrades all mRNA. This removes viral RNA from the cell, preventing its translation and the production of viral proteins (Clarke & Tyler, 2009).

Another well-studied receptor involved in innate immunity is TLR4, which recognizes the lipid A portion of LPS. This TLR is versatile in that it can induce signaling cascades leading to activation of transcription factors that promote transcription of both genes coding for type I IFNs and proinflammatory cytokines (Akira et al., 2006). This brings up an important point that although cytokines and type I IFNs can have specific effects on a target cell, such as the inhibition of transcription mediated by type I IFNs in response to viral infection, they more often have the tendency to mediate similar
effects on a variety of cells. For example, cytokines IL-6, IL-2, IL-4, IL-5, and IFNγ all mediate antibody production in B-cells (Kishimoto et al., 1994). This example also shows that cytokines play an important role in activating the adaptive immune response.

*The Role of NF-κB in the Innate Immune Response*

The signal transduction of all innate immune responses eventually converges at the point of NF-κB, a key transcription factor that directly controls the transcription of Type I IFNs and inflammatory cytokines. Its mechanism of action in differentiated cells is illustrated in Figure 4. In its inactive form, NF-κB is retained in the cytoplasm by its association with the inhibitor of NF-κB (IkB) (Liang et al., 2004).

**Figure 4**: NF-κB mediated expression of IFNs and inflammatory cytokines in differentiated cells

The first step toward NF-κB activation begins with an immune stimulus that activates a PRR, leading to the phosphorylation of IkB kinase (IKK). The signaling that occurs upstream of IKK phosphorylation varies based on the type of PAMP and PRR interaction initiating the signal. Once its kinase activity is activated, IKK phosphorylates
Phosphorylated IκB is then attached to ubiquitin (UUU), a small protein that signals the degradation of IκB. This frees the NF-κB dimer composed of a p50 and p65 subunit. The p65 subunit contains the conserved Rel homology domain where the nuclear localization signal is present. Degradation of IκB exposes this nuclear translocation signal, which induces nuclear translocation of NF-κB. Once it translocates into the nucleus, it initiates the expression of the genes encoding Type I IFNs (IFNα/β) and proinflammatory cytokines (Bonizzi & Karin, 2004; Liang et al., 2004) (Figure 4).

Innate Immunity in ESCs and their derivatives

Based on current literature, it appears that both ESCs and their derivatives are deficient in innate immunity as indicated by the low response of ESCs to pathogens and cytokines. mESCs were unable to appropriately respond to the LaCrosse Virus (LACV), as indicated by a lack in production of type I IFNs upon exposure to the virus, unlike naturally differentiated fibroblasts which greatly upregulate IFN expression (Wang et al., 2013). A follow up study, however, showed that mESCs were able to respond to exogenous IFNβ (Wang et al., 2014). Other studies found that both mESCs and their endothelial cell derivatives have low responsiveness to proinflammatory cytokines, specifically TNFα (Rajan et al., 2008), and hESCs and their derivatives were unable to appropriately respond to LPS and other pathogens (Foldes et al., 2010).

This low innate immune response in ESCs could be partially due to low expression of PRRs and downstream pathway components as has been reported in both ESCs and their derivatives. Wang et al. (2013) showed that there was no increased expression of virus-sensing PRRs such as RIG-I, MDA5, and TLR3 upon exposure of
mESCs to viral infection. Low levels of TLR3 and MDA5 were also reported in hESCs in comparison to HeLa cells. RIG-I was expressed in these cells, but many of its activators were expressed at low levels, while its inhibitors were highly expressed (Chen et al., 2010).

Although the innate immune dysfunction seems slightly improved with differentiation, the response is still deficient when compared to naturally differentiated cells. Since NF-κB is a universal transcription factor in the innate immune response as well as the converging point of many innate immune pathways, its dysfunction in these cells could be a molecular basis for this deficiency. A better understanding of the molecular basis of the lack of innate immune response in ESCs and their derivatives could contribute to the currently limited knowledge of innate immunity development. It could also lead to the direction of development of a differentiation method that does induce innate immune development, increasing the clinical applicability of ESC-DCs.
RATIONALE, HYPOTHESIS, OBJECTIVES

Due to their properties of pluripotency and self-renewal, ESCs are a promising source for regenerative medicine and cell therapy. Recent studies have demonstrated that mESCs can be differentiated to several cell types, including endothelial cells, smooth muscle cells, and neurons with expected morphology (Rajan et al., 2008; Guo et al., 2007). However, ESCs and their derivatives are not fully functional when compared to their in vivo counterparts; of particular concern is the recent finding that ESCs and their derivatives appear to have an attenuated immune response.

A recent study in our lab demonstrated that although susceptible to the cytopathic effect of viral infection, mESCs are deficient in expressing IFNα/β and cytokines, the hallmark of antiviral response and innate immunity (Wang et al., 2013). This study exemplifies that ESCs have a deficient innate immune response. Other laboratories have found similar results confirming this concerning deficiency in ESCs. Zampetaki et al. (2006) showed that mESCs have an underdeveloped immune response to bacterial challenges. It was also found that endothelial cells derived from ESCs have this deficiency (Zampetaki et al., 2006). These findings indicate a lack of developed innate immunity in ESC derivatives. This has led to the conclusion that current methods of differentiation are inadequate for promoting the development of innate immunity in vitro. If these cells lack a full immune response, they could be more susceptible to pathogens they encounter, potentially compromising the patient’s health.

A greater understanding of the molecular mechanisms controlling innate immunity could contribute to the establishment of a method to promote innate immunity development during in vitro differentiation. My project aimed to explain the molecular
basis of this innate immune deficiency, an aspect of this problem that has yet to be addressed. Based on the finding that ESCs fail to express IFNα/β and IL-6 in response to viral and bacterial challenge, I hypothesized that NF-κB was not activated by immune stimuli in ESCs, which may explain the failure of these cells to produce Type I IFNs and cytokines (Wang et al., 2013; Zampetaki et al., 2006). NF-κB seems like a likely candidate due to its role as a universal regulator of gene expression involved in the innate immune response. It controls the transcription of many immune response genes including type I IFNs and inflammatory cytokines (Liang et al., 2004). Considering endothelial cells derived from ESCs also had an attenuated immune response (Zampetaki et al., 2006), it is likely that NF-κB may not be fully functional in ESC-DCs. To test this hypothesis, I analyzed the function of the NF-κB pathway in ESCs and ESC-fibroblasts (ESC-FBs) and compared them with naturally differentiated fibroblasts where NF-κB is strongly activated. Although this study only focused on one of the many pathways involved in the innate immune response, a further understanding of this pathway could greatly contribute to the limited knowledge currently available about innate immunity development during differentiation. This could be instructive in obtaining ESC-DCs for clinical application.
EXPERIMENTAL DESIGN AND METHODS

Experimental design

To carry out this study, I used multiple cell types and several well-established molecular techniques. D3 cells, a commonly used mESC line, and fibroblasts derived from D3 cells (termed D3-FBs), were treated with a known activator of the NF-κB pathway, the proinflammatory cytokine TNFα. Naturally differentiated fibroblasts (C3H) were used as a positive control. Fibroblasts are an excellent model for the study of immunity due to their fully developed innate immune response, production of extracellular matrix, presence in most tissues, and the ease with which they can be differentiated from ESCs. Immunostaining was used to determine NF-κB’s subcellular localization, hence its activation. In stimulated cells, NF-κB will translocate into the nucleus of the cell where it can upregulate expression of targeted genes. The basal expression levels of receptors involved in inducing signal transduction leading to NF-κB activation were measured. The expression levels of NF-κB pathway receptors involved in viral recognition (RIG-I and TLR3), bacterial recognition (TLR4 and CD14), and cytokine recognition (TNFR2) were assayed by real-time quantitative polymerase chain reaction (RT-qPCR). The activation of NF-κB was further determined by the induction of gene expression under its control. The expression levels of the products of this pathway such as the proinflammatory cytokine IL-6 and the cellular adhesion molecules intracellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1) were assayed by RT-qPCR. The principles of the aforementioned techniques and methods are discussed below.
Cell Culture Techniques

Background

Cell culture is when cells are isolated from the tissue of an organism. Isolated cells are typically grown in a culture dish filled with media providing the appropriate environment and nutrients to support growth. Cell culture is a valuable technique that became common among research labs in the 1950s. The advent of antibiotics helped prevent contamination. The use of enzymes such as trypsin enabled dissociation of cells from their culture dish, allowing the continuous growth of transformed or continuous cell lines. Through further research, a defined medium able to more efficiently support cell growth was created. Finally, the commercialization of technology such as laminar flow hoods, aseptic disposable materials, and cell media made the use of cell culture commonplace (Ryan, 2008).

When cells are first obtained and grown in culture in a supportive artificial environment, this is called a primary culture. Eventually, the cells will fill the culture dish and must be subcultured. This requires the use of enzymes such as trypsin to carefully detach the cells from the culture dish, followed by replating of the cells at a lower density. Any extra cells can be stored in a cryoprotective agent such as dimethylsulfoxide or glycerol at temperatures below -130°C until later use (Ryan, 2008).

Another requirement for most cells is attachment factors. Most cells derived from normal tissues or organs are anchorage-dependent, meaning that they must be attached to a substrate in order to proliferate. To accommodate these cells, cell culture dishes are often coated with substances such as collagen or gelatin. The culture medium also must provide appropriate nutrients, maintenance of an appropriate pH (7.0-7.4), osmolality,
and growth factors and hormones (often supplied in the form of a 5-20% concentration of animal serum such as fetal bovine serum). Cells are stored at a temperature near the body temperature of the organism from which they were obtained, generally 37°C (Ryan, 2008).

**ESC Culture**

D3 and DBA252 cell lines were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 1000 U/mL of LIF, non-essential amino acids, 100U/mL penicillin, and 100μg/mL of streptomycin. Culture dishes were coated with 0.1% gelatin and stored in a humidified incubator at 37°C in an atmosphere at 5% CO₂. When the cells began to crowd the culture dish, they were subcultured. Briefly, trypsin, phosphate buffered saline (PBS), and DMEM containing 15% FBS were heated for 5-10 minutes at 37°C. A suction pipette was used to remove old medium. Enough PBS to cover the bottom of the dish was then added to wash the cells, and PBS was removed using a suction pipette. Trypsin was added to the culture dishes to gently detach the cells, and after cells were detached, an equal volume of 15% FBS DMEM was then added to inactivate the trypsin. Cells were then transferred to 1.5 mL tubes and centrifuged at 1,100 rpm for 3.5 minutes. A suction pipette was then used to remove the supernatant, and the cell pellet was resuspended in 1 mL of fresh medium. Cells were then replated to new gelatin-coated dishes at 50-60% confluence and maintained as described above.
**C3H10T1/2 Fibroblast Culture**

C3H10T1/2 cells (a fibroblast cell line isolated from 14 day mouse embryos, hereafter referred to as C3H) were cultured in DMEM containing 10% FBS, 100U/mL penicillin, and 100μg/mL of streptomycin. These cells were kept at 37°C in a humidifying incubator in an environment of 5% CO₂. The subculture procedure was identical to that of ESCs, except culture dishes were not coated with 0.1% gelatin as fibroblasts adhere well to plastic.

**In Vitro Differentiation of ESCs to fibroblasts**

mESCs (D3 or DBA cell lines) were seeded to 0.1% gelatin-coated tissue culture dishes and grown in a monolayer. The cells were treated with 1 μM retinoic acid (a vitamin A derivative that promotes differentiation) for 10 days, refreshing the medium every 3 days. This resulted in a mixed population of cell types including a large proportion of fibroblasts. The cells were then trypsinized and re-seeded to uncoated tissue culture dishes, and the medium was refreshed after 30-45 minutes to remove unattached cells. The remaining cells displayed typical fibroblast morphology and marker expression. These differentiated cells are referred to as either D3-FBs or DBA-FBs, depending on the ESC line from which they were derived. Fibroblast cells were maintained in the same conditions described for C3H cells (Wang et al., 2013).

These cells were differentiated into fibroblasts due to their role in innate immunity. Although fibroblasts are an important source of extracellular matrix proteins necessary to maintain connective tissue, they also play a major role in vascular inflammation. The main components of vascular inflammation are the activation of local endothelial cells and the recruitment of leukocytes circulating the blood to the site of
infection or inflammation. It appears that fibroblasts play a major role in this by acting as a virtual alert system, producing proinflammatory molecules upon exposure to a pathogen leading to activation of endothelial cells and recruitment of leukocytes (Enzerink and Vaheri, 2011).

**Immunostaining and Microscopic Analysis**

*Principle of Immunostaining*

Immunostaining is based on the principles of immunohistochemistry, which depends on antibody-antigen interactions with high affinity and avidity. Antibodies are produced by B-cells in the body in defense against invading pathogens. Antibodies are highly specific for foreign molecules known as antigens present on these pathogens. In immunostaining, an antibody specific for the protein of interest is used to treat fixed cells. This antibody is labeled with a fluorophore that will emit light when exposed to a certain wavelength of light under a fluorescent microscope (Koivunen & Krogstrup, 2006).

There are two methods used in immunostaining to fluorescently label the targeted molecule: direct and indirect. In the direct method, a single fluorescently labeled antibody made against the desired protein is used to label the desired protein. In the indirect method, two antibodies are used. The primary antibody is specific for the antigen of interest but is not fluorescently labeled. The secondary antibody, however, is fluorescently labeled and is specific to the primary antibody raised against the animal in which the primary antibody was raised. The indirect method allows for amplification of the fluorescent signal by limiting the possibility of cross reactivity by the fluorescently
labeled antibody and allowing multiple secondary antibodies to bind to a single primary antibody (Figure 5) (Koivunen & Krogsrud, 2006).

**Figure 5:** Comparison of direct method of immunostaining (I) and indirect method of immunostaining (II) (adapted from Koivunen & Krogsrud, 2006).

**Immunostaining Protocol**

Cells were fixed with methanol for 15 minutes. Methanol causes denaturing of cellular proteins, allowing for entry of antibodies into the cell. Fixation also maintains the cell structure and integrity after biological processes have halted. Methanol was then removed, and PBS was added allowing cells to be stored at 4°C until needed. Blocking buffer composed of 2% bovine serum albumin (BSA) in Tris-buffered saline with Tween-20 (TBST) was then added to the cells for thirty minutes. The blocking step prevents non-specific interactions of antibodies therefore limiting background staining. The cells were then incubated with a primary antibody raised against the targeted molecule in a 1:100 dilution in 2% BSA for two hours at room temperature. The cells were then washed in TBST three times for two minutes each to remove any free, excess antibody. The cells were then incubated with the fluorescently labeled secondary antibody specific for the primary antibody. This incubation was left in the dark at room temperature for
two hours. The cells were then washed in TBST three times again. 20 μL of mounting medium was placed on a new, appropriately labeled microscope slide. The coverslips were then placed on the mounting medium drops, cell side down. Two coats of nail polish were applied to seal the coverslips on the slides. The slides were then stored in the dark at 20°C until viewed. Slides were examined using a Zeiss LSM 5-10 laser scanning confocal microscope. The obtained images were analyzed using Zeiss LSM Image Examiner Software.

**Immunostaining to Detect Nuclear Translocation of NF-κB by Confocal Microscopy**

ESCs, C3H cells, D3-FBs, and DBA-FBs passaged 8 times (p8) were cultured following the cell culture protocol. One set of each cell type received no treatment to act as a control. Cells were grown on cover glasses which were kept in a 24 well container in 0.5 mL of 15% FBS DMEM until they reached 100% confluence. One set of each cell type was switched to 2% FBS medium for about 15 hours prior to a 15-minute treatment of TNFα at a concentration of 20ng/mL. Control cells from each cell type (C3H, D3-FBs, and DBA-FBs) and cells treated with TNFα 15’ from each cell type were blocked using 2% BSA. They were then treated with the primary antibody- polyclonal rabbit anti-NF-κB p65 (Santa Cruz, Biotechnology) diluted to a concentration of 1:100 in 2% BSA for about two hours. After washing with TBST, these cells were treated with the secondary antibody- goat anti-rabbit IgG-Texas Red: (Santa Cruz, Biotechnology,) diluted to a concentration of 1:100 in 2% BSA for about two hours. These cells were then mounted using mounting medium and viewed on the fluorescent confocal microscope using the laser at the wavelength of 543nm (rhodamine/Texas red).
**Immunostaining to Characterize ESC-FBs**

Four untreated cell samples of D3-FBs were blocked using 2% BSA. One cell sample was treated with the primary antibody- polyclonal rabbit anti-NG2 chondroitin sulfate proteoglycan IgG at a concentration of 1:100 in 2% BSA for about 2 hours. This culture was then treated with a secondary antibody- goat anti-rabbit IgG-FITC at a concentration of 1:100 in 2% BSA for about 2 hours (Santa Cruz, Biotechnology). One culture was treated using the same conditions and antibody concentrations with the primary antibody- goat anti-type IV collagen polyclonal antibody (Santa Cruz, Biotechnology) and a secondary antibody- goat anti-goat IgG-FITC (Santa Cruz, Biotechnology). Another culture was treated using the same conditions and antibody concentrations using a primary antibody- Mouse Anti-MMP-14 [MT1-MMP] monoclonal antibody (Santa Cruz, Biotechnology) and a secondary antibody goat anti-mouse IgG-FITC (Sant Cruz, Biotechnology). The other cell culture was treated with a primary antibody- mouse anti-Ncadherin (Santa Cruz, Biotechnology) and a secondary antibody- goat anti-mouse IgG-FITC (Santa Cruz, Biotechnology). These cells were mounted using mounting medium with DAPI and later viewed on the fluorescent confocal microscope using the following lasers: 405 nm (DAPI) and 488 nm (FITC). DAPI staining allows for visualization of the nucleus.
RNA Extraction and RT-qPCR

*Principle of PCR-based Gene expression analysis*

RT-qPCR allows for the measure of DNA amplification as it occurs due to the presence of a molecule that fluoresces when it binds to double stranded DNA. As DNA is amplified and double stranded DNA accumulates so does fluorescence. A common fluorescent DNA binding chemical used is SYBR green. As shown in Figure 6, there are four phases that occur during RT-qPCR: ground phase, early exponential phase, log-linear phase, and plateau phase. During the ground phase, amplification is just beginning and enough double stranded DNA is not yet present to produce a detectable increase in fluorescence. During early exponential phase, a high enough concentration of double stranded DNA is present to produce a detectable increase in fluorescence. The cycle at which early exponential phase occurs is referred to as the Ct, which is indicated by the red arrow in Figure 6. The Ct value is assumed to be equivalent to the amount of copy DNA originally in the sample. During the log linear phase, the DNA within the sample undergoes its most efficient replication, ideally doubling the amount of DNA present with every cycle. During the plateau phase, DNA replication begins to slow down and eventually end due to depletion of necessary resources such as nucleotides (dNTPs).

Using the Ct values, the relative expression of the genes of interest can be determined by comparison to a housekeeping gene, a gene that is constitutively expressed in the cells being tested. These are genes such as β-actin and GADPH. A commonly used method of analysis is the comparative Ct method, which uses a formula to find the fold change using a ratio between the relative expressions of the gene of interest compared to the housekeeping gene. The formula is as follows (Wong & Medrano, 2005) (Pfaffl, 2001):
\[
\frac{\text{expression in experimental group}}{\text{expression in control group}} = \frac{2^{(C_t \text{ ref} - C_t \text{ gene of interest})_{\text{experimental}}}}{2^{(C_t \text{ ref} - C_t \text{ gene of interest})_{\text{control}}}}
\]

**Figure 6:** Phases of RT-qPCR. The \( C_t \) value is indicated by the red arrow (adapted from Wong & Medrano, 2005)

**RNA Extraction**

RNA was isolated from cells using Sigma Tri-reagent. 0.6 mL of Tri-reagent was added per 28cm\(^2\) area of culture dish; Tri-reagent was added directly to culture. The cells were then collected and transferred into 1.5 mL microfuge tubes. 0.2 mL of chloroform/mL of Trizol was added and the tube was vortexed for 15 seconds. The sample was then incubated at room temperature for 10 minutes. The sample was then centrifuged at 10,000 rpm for 10 minutes at 4\(^\circ\)C. This led to a phase separation. The RNA was present in the aqueous phase, which was removed and placed in a new tube. The protein was in
the interphase or flocculent as it was attracted to both the organic and aqueous phases due to the amphipathic nature of proteins. The DNA migrated to the organic phase or flocculent due to the acidic environment and presence of phenol: chloroform. The H+ neutralizes the phosphate groups on DNA making it without charge and therefore nonpolar and attracted to the organic and interphase. 0.5 mL of 100% isopropanol was added per mL of Tri-reagent used for homogenization (0.5 mL if 1 mL Tri-reagent used). The sample was left to incubate at room temperature for ten minutes. Following incubation, the sample was centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was removed from the tube, leaving only an RNA pellet. The pellet was washed with 1 mL of 75% ethanol per 1 mL of Tri-reagent used in initial homogenization (1 mL used if 1 mL Tri-reagent used). The sample was left in the -20°C freezer for at least one hour to allow for RNA precipitation. The sample was centrifuged at 1200 rpm for 15 minutes at 4°C and the ethanol was discarded. The RNA pellet was left to air dry for 5-10 minutes on ice without allowing the pellet to be over dried. The pellet was resuspended in DEPC water. The concentration and integrity ($A_{260}/A_{280}$ was between 1.8-2.2) of RNA was determined using a spectrophotometer. The sample was stored at -70°C until later use.

**Reverse Transcription Protocol**

In order to generate the cDNA to be quantified by PCR amplification, reverse transcription of total RNA was performed. The RNA sample was then reverse transcribed using M-MLV reverse transcriptase. The RNA was diluted in DEPC water to an 11.5μL total volume at 1μg/μL. 1μL of dNTPs (10μM) and 2μL of random hexamers primer (10X) were added to the sample. The sample was then incubated at 70°C for five
minutes then stood on ice. 4μL of 5X M-MLV buffer, 1μL of M-MLV reverse transcriptase, and 0.5mL of RNase inhibitor were added to the sample. The sample was incubated in the thermal cycler at 42°C for one hour, then 10 minutes at 95°C to inactivate the enzyme. 200μL of DEPC water was added to the now cDNA sample which was stored at -20°C until needed.

**Quantitative PCR protocol**

A 20 μL volume reaction was made for each sample composed of: 10 μL of 2X SYBR mix, 3 μL of DEPC water, 5 μL of cDNA and 2 μL of the appropriate primer (a primer specific for the gene of interest). The samples were then analyzed using qPCR that was run for 40 cycles. The data was analyzed using the comparative C<sub>t</sub> method and all data was relative to β-actin expression (Pfaffl, 2001). The sequence of the β-actin primers were as follows:

Forward Primer: 5'-CATGTACGTAGCCATCCAGGC-3'
Reverse Primer: 5'-CTCTTTGATGTCACGCACGAT-3'

Analysis provided the relative RNA levels, and therefore expression level, of the gene of interest.

**Determination of PRR Expression levels**

RT-qPCR was used to determine the basal level of expression of common PRRs in p5 D3-FBs and C3H cells. The following primers were used to test the expression levels of the following PRRs:
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIG-1</td>
<td>5’-ATTCAGGAAGAGCCAGAGTGTC-3’</td>
<td>5’-GTCTTTCAATGATGTGCTGCAC-3’</td>
</tr>
<tr>
<td>MDA5</td>
<td>5’-CGATCCGAATGATTTGATGCA-3’</td>
<td>5’-AGTTGGTCATTGCAACTGCT-3’</td>
</tr>
<tr>
<td>TLR3</td>
<td>5’-CTTGCCTTGGGGAAGTGCAAGA-3’</td>
<td>5’-CCAATTGTCTGGAAACACCC-3’</td>
</tr>
<tr>
<td>TLR4</td>
<td>5’-TGCACTGCTTTAGTGTTGCT-3’</td>
<td>5’-GACCCATGAAATTGGCACTCA-3’</td>
</tr>
<tr>
<td>CD14</td>
<td>5’-CTCTGTCCTTAAAGCGGCTTAC-3’</td>
<td>5’-GTTGCGGAGGTTCAAGATGTT-3’</td>
</tr>
<tr>
<td>TNFR2</td>
<td>5’-GCCCAGGTTGTCTTGACA-3’</td>
<td>5’-CACAGCACATCTGAGGCTTCC-3’</td>
</tr>
</tbody>
</table>

**Determination of NF-κB Targeted Gene Expression Level**

RT-qPCR was used to determine the expression of TNFα induced NF-κB targeted genes. D3-FBs (p9) and DBA-FBs (p10) and C3H cells were treated with 20 ng/ml TNFα in 2% FBS DMEM for 24h. The following primers were used to test the expression levels of the following NF-κB targeted genes:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1</td>
<td>5’-GGCATTTGTCTCTAATGTCTGGC-3’</td>
<td>5’-GCTCCAGGATATGCTCCTTC-3’</td>
</tr>
<tr>
<td>VCAM</td>
<td>5’-CCAAATCCACGCTTGTGTTGA-3’</td>
<td>5’-GGAATGAGTAGGCACCTCCC-3’</td>
</tr>
<tr>
<td>IL-6</td>
<td>5’-TAGTCCCTTCTACCCCAATTCC-3’</td>
<td>5’-TTGGCTCTTAGCCACTCCTTC-3’</td>
</tr>
</tbody>
</table>
RESULTS

Characterization of ESC-FBs

Fibroblasts play a major role in the innate response, especially in vascular inflammation. These cells produce proinflammatory molecules upon exposure to a pathogen leading to activation of endothelial cells and recruitment of leukocytes. Fibroblasts produce many molecules important to the immune response, including cytokines such as interleukins and interferons (Enzerink & Vaheri, 2011). They also produce cellular adhesion molecules such as ICAM-1, important in recruiting leukocytes to the site of infection (Zittermann & Issekutz, 2006). Murine embryonic fibroblasts (MEFs) have also been shown to highly express receptors, such as TLRs and have been used to characterize these receptors (Kurt-Jones et al., 2004). Together, all of these qualities make fibroblasts an ideal model for the study of innate immunity.

Fibroblasts were differentiated from ESCs by the method utilizing retinoic acid. To confirm the mESC-FBs were in fact fibroblasts, they were first compared to the naturally differentiated fibroblast cell line C3H, which are derived from early mouse embryos (Wang et al., 2013). Using the toluidine blue stain, the morphologies of C3H cells and D3-FBs were compared. This data is shown in Figure 7A. D3 ESCs exhibited typical ESC morphology. The cells had a round shape with small cell bodies in culture. However, the D3-FBs exhibited morphology similar to C3H cells. They had a more elongated and flattened cell body. In addition to morphology, some fibroblast markers were expressed in D3-FBs as detected by immunostaining and confocal microscopy. The
markers used were: neural cadherin (NCAD), neural antigen 2 (NG2), and collagen 4 (Col4) (Figure 7B).

Figure 7: Characterization of ESC-FBs. A: Toluidine blue staining of methanol fixed D3-ESCs, C3H cells, and D3-FBs. B: Fibroblast markers were analyzed using immunostaining in D3-FBs. The indicated proteins were identified with their specific antibodies and detected with secondary antibodies labeled with FITC (green). The nucleus was stained with DAPI (blue).

Expression of PRRs in ESCs, D3-FBs, and C3H cells as Measured by RT-qPCR

Since the attenuated immune response observed in ESCs could be due to a low expression of PRRs, leading to an inability of these cells to recognize and respond to pathogens, the expression levels of PRRs involved in viral, bacterial, and cytokine recognition were analyzed in ESCs, D3-FBs, and C3H cells. These PRRs should be highly expressed in fibroblasts considering MEFs have been shown to highly express TLRs (Kurt-Jones et al., 2004). Increased expression of these receptors in ESC-FBs could indicate increased functionality of the innate immune response following differentiation.
As shown in Figure 8, expression of all PRRs in ESCs was extremely low when compared to expression levels in D3-FBs and C3H cells. However, this expression increased following differentiation, although most PRRs were still not expressed as highly as in naturally differentiated C3H cells. The only PRRs that were expressed at similar levels to C3H cells in D3-FBs were TNFR2, CD14, and MDA5.

**Figure 8:** Basal PRR expression in ESCs, D3-FBs, and C3H cells. RT-qPCR in p5 D3-FBs and C3H cells determined the basal mRNA levels of several PRRs. The values are means ± SD of three independent experiments.

**Determination of NF-κB Nuclear Translocation by Immunostaining and Microscopic Analysis**

A well-established method to determine NF-κB activation is by immunodetection of its cellular location. In unstimulated cells, NF-κB is mainly detected in the cytoplasm. TNFα treatment induces the translocation of NF-κB to the nucleus. This method was
utilized to determine the functionality of the NF-κB pathway in ESCs, ESC-FBs, and C3H cells. Since NF-κB is a universal regulator of many genes involved in the innate immune response, its functionality is a good reflection of the functionality of the innate immune response itself. Therefore, this experiment sought to determine if there was increased NF-κB activity, and therefore a more developed innate immune response in ESC-DCs.

Treated cells were first incubated with anti-NF-κB antibodies followed by a treatment of a secondary, rhodamine-conjugated antibody specific for the anti-NF-κB antibody, and analysis by fluorescence confocal microscopy. The results, shown in Figure 9, confirm that TNFα did not induced nuclear translocation of NF-κB in ESCs since the fluorescence is seen entirely in the cytoplasm in both TNFα treated and control ESCs. This correlates with the previous results (Figure 8) showing low expression of PRR receptors in ESCs. These results indicate that NF-κB is not functional in ESCs.

Although expression of PRRs is generally lower in ESC-FBs than in naturally differentiated C3H cells (Figure 8), it seems that nuclear translocation of NF-κB is functional. This can be seen by the fact that fluorescence is detected in the nucleus of D3-FBs upon treatment with TNFα rather than in the cytoplasm as seen in the control. This is the same as the results in C3H cells. This result was confirmed in DBA-FBs.
Figure 9: TNFα induced NF-κB translocation. ESCs, D3-FBs, DBA-FBs, and C3H cells were treated with TNFα (20ng/mL) for 15’ in 2% FBS DMEM and stained for NF-κB. The subcellular location of NF-κB was then visualized by confocal microscopy (mag=400X).

**TNFα Induced Expression of NF-κB regulated Genes in ESCs, C3H and ESC-FBs**

Following nuclear translocation of NF-κB, it acts as a transcription factor, binding to the promoters of many genes important in the innate immune response, and upregulating their expression by increased transcription. To determine the functionality of translocated NF-κB at upregulating expression of its targeted genes, the expression levels of these genes in TNFα treated cells were determined.
Using RT-qPCR, the expression levels of ICAM-1, VCAM, and IL-6 by TNFα treatment in cells were determined. These results are shown in Figure 10. Despite the functionality of NF-κB nuclear translocation in ESC-FBs, the upregulation of NF-κB targeted gene ICAM-1 in response to TNFα was lower than in naturally differentiated C3H cells. However, the mRNA fold change of VCAM and IL-6 were relatively similar in all cell types, which were low in all cells tested. ICAM-1 expression was increased about 100 fold in C3H cells, but it was only approximately increased by 10 fold in D3-FBs and DBA-FBs. This is about one fifth of the fold change seen in C3H cells.

**Figure 10:** The induction of ICAM-1, VCAM, and IL-6 mRNA by TNFα treatment was determined by RT-qPCR at 24 h post treatment. D3-FBs (p9) and DBA-FBs and C3H cells were treated with 20 ng/ml TNFα in 2% FBS DMEM for 24h. The results are expressed by mRNA fold change, and mRNA levels in the control cells are designated as 1. The values are means ± SD of two independent experiments.
DISCUSSION

This study sought to obtain a better understanding of the molecular mechanism underlying the attenuated innate immune response to viruses and cytokines in mESCs and their derivatives. While it is understood that mESCs and their derivatives have an attenuated immune response, the underlying molecular mechanisms are unclear. Since innate immune signaling eventually converges at the point of NF-κB, my project focused on understanding if a defect in this pathway is contributing to this dysfunction in mESCs and their differentiated cells in response to TNFα.

Based on the finding that ESCs fail to express IFNα/β and IL-6 in response to viral and bacterial challenge (Wang et al., 2013), it was hypothesized that NF-κB was not activated by immune stimuli in ESCs, offering an explanation for the failure of these cells to produce Type I IFNs and cytokines. Since the derivatives of mESCs were also unable to mount an appropriate immune response to TNFα (Rajan et al., 2008), it was also postulated that the NF-κB pathway was defective in mESC derivatives as well. Confirmation of these findings would indicate differentiation conditions were inefficient at upregulating NF-κB pathway development.

In this study, the function of the NF-κB pathway in ESCs and ESC-DCs was analyzed. ESC-FBs were specifically used due to the role of fibroblasts in tissue inflammation and their sensitivity to the stimulation of cytokines such as TNFα (Enzerink and Vaheri, 2011; Kurt-Jones et al., 2004).
The first aspect of the NF-κB pathway examined was the pathway receptors. Since ESCs and their derivatives seem to be defective in responding to microbes, viruses, and cytokines, receptors recognizing each of these were selected. The expression levels of receptors recognizing dsRNA PAMP of viruses (RIG-I, MDA5, and TLR3) were barely expressed in mESCs (Figure 8). This is expected based on previous studies showing that these three PRRs were not expressed even when exposed to LACV (Wang et al., 2013). Although there was some increase in expression of these PRRs after differentiation, the expression was lower than in C3H cells. This pattern remained constant for TLR4 (a receptor recognizing LPS of bacteria) expression as well, which is in agreement with previous studies (Foldes et al., 2010; Zampetaki et al., 2006).

However, previous studies had shown TNFR2, a receptor recognizing TNFα, was expressed at low levels in mESCs and their retinoic acid differentiated derivatives when compared to naturally differentiated cells (Kim et al., 2008). This result was confirmed in hESCs and their retinoic acid differentiated derivatives (Kang et al., 2007). This, however, was not a reflection of our results. Our results indicated that retinoic acid induced differentiation to fibroblasts (D3-FBs) was enough to induce TNFR2 expression to the same levels of C3H cells. This was the same pattern seen in CD14 expression, another bacterial PRR. This inconsistency could be due to the use of different cell lines in these two studies.

Low basal expression of pathway receptors in mESCs could be a great contributor to innate immune dysfunction. This low expression could prevent the necessary PRR/PAMP interaction to initiate activation of the NF-κB pathway. This activation was determined by detection of the subcellular location of NF-κB by immunostaining and
microscopic analysis. As shown in Figure 9, when treated with TNFα, mESCs retained NF-κB in the cytoplasm, indicating the pathway was not activated by cytokine exposure. However, the mESC-FBs (D3-FBs and DBA-FBs) showed nuclear translocation of NF-κB upon exposure to TNFα similar to the nuclear translocation seen in C3H. These results seem to indicate that despite the lower expression of most TLRs seen in mESC-FBs (D3-FBs and DBA-FBs) in comparison to C3H cells, the NF-κB pathway seems to become functional following differentiation. This would indicate that differentiation alone is enough to induce innate immunity development at least in regards to NF-κB nuclear translocation.

However, when the expression levels of NF-κB regulated genes in response to TNFα treatment were examined by RT-qPCR, the induction of the gene ICAM-1 in the mESC-FBs (D3-FBs and DBA-FBs) was significantly lower than in C3H cells. As shown in Figure 10, the mRNA fold change of ICAM-1 in mESC-FBs was one fifth that of the fold change seen in naturally differentiated fibroblasts. Expression of this molecule is strongly induced by TNFα treatment in fibroblasts (Shan et al., 2010). While expression of VCAM and IL-6 seemed to be relatively consistent throughout all cell types, the response was less than ten fold in all cell types as well, indicating that these particular cells may just weakly upregulate these genes in response to TNFα.

The results indicate that the dysfunction of the NF-κB pathway seems to occur at the steps downstream of NF-κB translocation, specifically in upregulation of ICAM-1. This raises the question: why is NF-κB not promoting gene expression of ICAM-1? It can be speculated that the ICAM-1 promoter activity is mediated by epigenetic
modifications. This would be similar to what Zampetaki et al. (2006) found in relation to the TLR4 promoter. They discovered that this promoter was methylated in ESCs and their endothelial derivatives, which prevents transcription. Another possible explanation is that there is some sort of defect in the NF-κB binding activity, preventing it from appropriately binding to the ICAM-1 promoter.

Overall, my results confirmed the finding of many others that the NF-κB pathway is not functional in mESCs, but is induced to some extent by current differentiation methods, as exemplified by the NF-κB nuclear translocation data. However, the functionality of the NF-κB pathway is not equivalent to that found in their naturally differentiated counterparts. This deficiency is likely due to differentiation methods that do not provide the specific environment or factors required to induce complete activation of NF-κB regulated gene expression. Since more than one transcription factor is often involved in the transcriptional activation of a gene, it could be a dysfunction in another pathway contributing to this attenuated ICAM-1 expression. For example, the transcription factor AP-1, activated by the MAP-kinase pathway appears to be involved in regulating innate immune gene expression (Rajan et al., 2008). At the present time, it is not clear if this pathway is functional in ESCs and ESC-DCs.

Although more research needs to be conducted to understand the exact molecular mechanisms underlying the innate immune deficiency seen in ESCs and their derivatives, it seems that NF-κB dysfunction, downstream of its nuclear translocation, may play a role in this mechanism. Although this study only focused on some aspects of the NF-κB pathway’s involvement in the innate immune response, a further understanding of this pathway and its role in the development of innate immunity could greatly contribute to the
limited knowledge currently available about innate immunity development in ESCs. Through further experimentation, I expect that a novel differentiation strategy will be able to generate ESC derivatives with an activated NF-κB pathway, making their potential for use in regenerative medicine even greater.
REFERENCES


