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Mouse Embryonic Stem Cells Are Sensitive to the Cytotoxicity of Nitric Oxide: Biological Implications for Early Embryogenesis

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by

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Abstract

The early embryo, before implantation, is at a very vulnerable stage in development where it faces various inflammatory cytokines throughout the implantation process. In this stage, the cells in the blastocyst, the preimplantation stage embryo, must proliferate rapidly for tissue formation. However, it is known that inflammatory cytokines can inhibit cell proliferation. Previous studies have shown that embryonic stem cells (mESCs), the major cell component in the blastocyst, are unresponsive to treatments of tumor necrosis factor α (TNFα) and interferon γ (IFNγ), two inflammatory cytokines involved in the implantation process. Treatment of mESC-differentiated fibroblasts (mESC-FBs) with TNFα and IFNγ in combination (TNFα/IFNγ) significantly reduced cell viability and the rate of cell proliferation; however, this treatment has no effect on the cell viability and the cell cycle of mESCs. It has been previously demonstrated that inducible nitric oxide synthase (iNOS) stimulated by TNFα/IFNγ is responsible for the effects of TNFα/IFNγ, since NO produced by iNOS is a free radical that can cause cellular damage. Based on this finding, it is hypothesized that the resistance of mESC to TNFα/IFNγ cytotoxicity is due to their lack of response to these two cytokines, therefore, iNOS and NO were not produced. This allows mESCs to avoid the cytotoxicity of TNFα/IFNγ. To test this hypothesis, sodium nitroprusside (SNP), a NO donor, was used to determine the sensitivity of mESCs to NO. SNP treatment resulted in decreased cell viability through increasing apoptosis, suggesting that mESCs are susceptible to the cytotoxicity caused by NO. Therefore, the lack of iNOS induction by TNFα/IFNγ in mESCs may help to protect mESCs from the cytotoxicity of the two cytokines at the early stage of embryogenesis.

Keywords: mESC, innate immunity, iNOS, SNP, inflammatory cytokines

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APC - antigen presenting cell
cDNA - complementary DNA
DAPI - 4′,6-diamidino-2-phenylindole
DMEM - Dulbecco's modified Eagle medium
eNOS - endothelial nitric oxide synthase
ESC - embryonic stem cell
ESC-FBs - embryonic stem cell-differentiated fibroblasts
FBS - fetal bovine serum
hESC - human embryonic stem cell
ICM - inner cell mass
IFN - interferon
IFNγ - IFN gamma
iNOS - inducible nitric oxide synthase
iPSC - induced pluripotent stem cell
JAK - Janus tyrosine kinase
LIF - leukemia inhibitory factor
LPS - lipopolysaccharide
mESC - mouse embryonic stem cell
MHC - major histocompatibility complex
M-MLV - Moloney Murine Leukemia Virus
NF-κB -nuclear factor-κB
nNOS - neuronal nitric oxide synthase
NO - nitric oxide
PAMP - pathogen associated molecular pattern
PBS - phosphate-buffered saline
PCR - polymerase chain reaction
PRR - pattern recognition receptor
ROS - reactive oxygen species
RT-qPCR - quantitative real-time PCR
SDS - sodium dodecyl sulfate
SNP - sodium nitroprusside
STAT - signal transducers and activators of transcription
TB - toluidine blue
TLR - toll-like receptor
TNFα - tumor necrosis factor alpha
TNFα/IFNγ - TNFα and IFNγ in combination

CHAPTER ONE: INTRODUCTION

Embryonic Stem Cells

Embryonic Stem Cells Isolation and Characterization

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass (ICM) of blastocyst-stage embryos (Keller, 2005). Pluripotency gives these cells the ability to differentiate into cell types of all three germ layers: ectoderm, mesoderm and endoderm, as seen in Figure 1. As they differentiate into cell types of the germ layers, they gain functions of the cell type and lose the ability to differentiate into other cell types. ESCs also have the capacity of self-renewal: they can divide indefinitely in cell culture as long as they have the appropriate growth factors (Keller, 2005). All types of stem cells have potency and self-renewal; however, there are different levels of potency. Adult stem cells, existing in certain tissues of developed organisms, are mostly considered multipotent, meaning they can differentiate into a subset of cell types with limited self-renewal capacity. An example of such cells is Hematopoietic Stem Cells, which give rise to cells of the blood including erythrocytes and leukocytes. While they give rise to cells of the blood, they cannot be differentiated into any other cell types (Cao et al., 2013). In contrast,

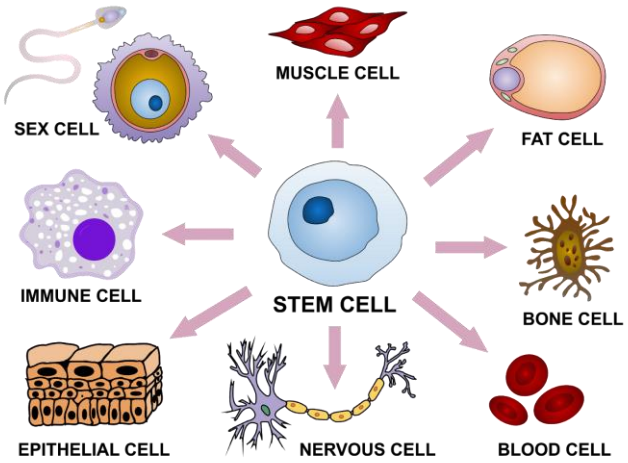

Figure 1: Overview of ESC Differentiation (image from "Cellular differentiation," 2020)

some adult stem cells are considered unipotent, meaning they can differentiate into only one cell type. In addition to multipotency and unipotency, zygotes are considered totipotent cells, meaning they can differentiate into any cell type including extraembryonic tissues. (Mitalipov & Wolf, 2009).

During development, the embryo goes through stages. Once an egg is fertilized by sperm, the resulting cell is known as a zygote. The zygote quickly goes through a process of cleavage in order to produce more cells. This process involves the cell going through several rounds of mitosis. Once the zygote goes through several divisions and reaches the 8-cell stage, it is known as a morula, or a solid sphere of cells. Totipotency is maintained up until this point. Further divisions known as blastulation results in the cell forming the blastocyst, a hollow ball of cells filled with fluid. At this point, the first cell fate decision has occurred where the cells have specialized towards a prospective trophoblast fate; the decision is based on cell polarity, cell signaling and transcription factors (Kubaczka et al., 2017). The outer cells are known as the trophectoderm, while the cells on the inside are called the ICM (Kubaczka et al., 2017). The ICM develops into the embryo, while the trophectoderm develops into the placenta. This process, in humans, lasts 8-9 days. While the ball of cells is dividing, it is also making its way through the fallopian tube and into the uterus, where it is implanted at the blastocyst stage.

Mouse ESCs (mESCs) are isolated from the ICM of the blastocyst. The mESCs are then cultured with a specific cell medium in order to maintain their pluripotency. In the past, mESCs were co-cultured with a layer of mouse embryonic fibroblasts which served as feeder cells (Wobus & Boheler, 2005). It was believed that the feeder cells were needed in order to maintain the mESCs undifferentiated state; however, with further study, it was determined that leukemia inhibitory factor (LIF) was the key source in preventing differentiation and maintenance of the

stem cell state of mESCs (Wobus & Boheler, 2005). The cytokine LIF acts through the signal transducers and activators of transcription (STAT) pathway by activating Stat3 (Wobus & Boheler, 2005). While this is effective in mESCs, the activation of Stat3 is not enough to maintain the stem cell state in human ESCs (hESCs), showing a clear molecular difference between the experimental models (Wobus & Boheler, 2005).

Ethical and Social Concerns

Using hESCs in research has become controversial due to the idea that a blastocyst has to be sacrificed in order to obtain hECSs. While the blastocyst can become a life, it must be implanted into a uterus and carried to term. It is not possible for a baby to be fully formed in vitro. More importantly, hESCs used in research are mainly obtained from in vitro fertilization, not directly isolated from naturally developed blastocysts. In vitro fertilization often results in an excess of fertilized eggs. Rather than throwing them away, these eggs can be used for derivation of hESCs and have the potential to save many lives through regenerative medicine. While this is more ideal than throwing them away, some people see this as the ending of a potential life. The Bush administration, in 2001, put into place an executive order that limited federal funding to only hESCs lines that were in existence, which included funding from the National Institute of Health (NIH) (Hyun, 2010). However, Barack Obama, in 2009, revoked this previous executive order to allow the NIH to fund hESC studies as long as the studies are conducted responsibly and are scientifically worthy (Executive Order No. 13505, 2009).

Because hESC research can be very controversial, a lot of research is done on mESCs. There are fewer ethical restrictions on mice, so many researchers use these as a model instead of hESCs. While mice serve as adequate experimental models, there are many unknowns as to how similar the cell types are to humans. In addition to using mESCs, there has also been research on

induced pluripotent stem cells (iPSCs) which involve fewer ethical restrictions. These are cells, usually fibroblasts, that were differentiated; however, they are induced to go back to the pluripotent stage through the use of master transcription factors such as Oct4 and Sox2 (Hyun, 2010). Once back in the pluripotent stage, they have the ability to differentiate into any cell type like ESCs. This could be very beneficial to bypassing the ethical concerns that hESCs carry with them. In addition to this, iPSCs, while manipulated, still maintain cell components recognizable by the donor's own immune system, reducing the potential for rejection. However, many scientists do not believe iPSCs should replace hESC studies (Hyun, 2010).

Potential Applications in Regenerative Medicine

Because ESCs have high potency and multiple applications, as illustrated in Figure 2, scientists believe they should be studied. Because they can be differentiated into cells of all three germ layers, they facilitate research on the effects of treatment, medicines and cytotoxicity that could not be studied in vivo. ESCs allow for the possibility of cell replacement therapy, especially to help in repairing cell types of the body that are unable to renew. Examples of such cells are neurons and cardiac muscle cells. If cardiac muscle cells are damaged from a heart attack, necrosis of heart tissue can occur. While these cells do not have the capability of regenerating, with stem cell treatments, there is hope that ESC-differentiated cells could help replace the damaged tissue. This would allow for restoration of function of the damaged tissues and organs known as regenerative medicine. The hope is that previously untreatable conditions may become treatable through the use of ESCs.

One of the issues with ESCs in regenerative medicine is that if ESCs are not differentiated fully and transplanted into a patient, tumor-like structures can form because the cell cycle may not be regulated (Hentze et al., 2009). Obviously, this is a huge risk to the

patient's health. There is also a possibility that once transplanted, these cells will not function properly in organs (Wobus & Boheler, 2005). In addition, there are many unknowns as to how the ESCs' innate immunity will react when they are exposed to infectious agents (Wang et al., 2013). ESCs' innate immunity is not well known, but for the few studies that have been completed, ESC derived cells have limited ability to respond to lipopolysaccharide (LPS), a component of gram-negative bacterial cell membranes, and inflammatory cytokines (Guo et al., 2015). While ESCs lack a functional innate immunity, it is unknown if the deficiency is beneficial or detrimental to regenerative medicine applications. Lacking strong inflammatory responses to the transplanted area could be beneficial by reducing damage to the grafted cells (Guo et al., 2015). However, if there are infections, the cells may fail to mobilize the adaptive immune system, thus failing to react effectively. Overall, the lack of ESC derived cells to acquire a fully functional innate immunity could be a concern for applications in the clinical setting (Guo et al., 2015).

ESCs as a Tool to Study Embryogenesis and Developmental Biology

In addition to the use of ESCs in regenerative medicine, this cell type has allowed for the ability to study embryogenesis and developmental biology, as seen in Figure 2. The properties of ESCs can be studied to determine the molecular mechanisms of the cell type. This is especially important because they have unique characteristics compared to differentiated cell types (Keller, 2005). Part of the ability to study mESCs is maintaining their pluripotency and undifferentiated cell state by using LIF in the cell culture (Keller, 2005). In order to test to see if the cells have maintained their pluripotency, besides checking for differences in cell morphology, the transcription factors Oct4, Sox2, and Nanog can be tested (Schnerch et al., 2010). These are key

modulators in maintaining pluripotency which is evident in the production of iPSCs. If LIF is removed from the ESC culture, differentiation occurs.

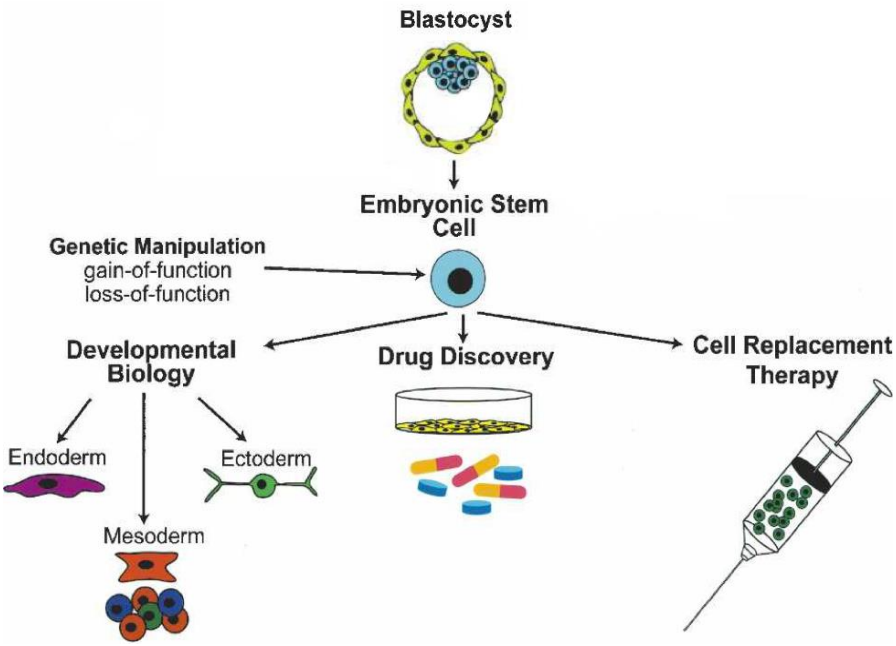

Figure 2: Overview of Potential Uses of ESCs in Basic Research and Medicine (Image adapted from Keller, 2005)

When LIF is removed, ESCs differentiate spontaneously. In addition to spontaneous differentiation, there is directed differentiation to specific somatic cell populations through the use of different growth factors or cytokines, but these techniques are inefficient and result in a population of multiple cell types (Wobus & Boheler, 2005). With further studies using ESCs to determine mechanisms involved in the differentiation process, embryogenesis and development from the blastocyst stage into the fully formed organism will be better understood. In addition, the formation of cells from all three germ layers may allow these cell types to be studied in vitro.

Immune and Inflammatory Responses as Cellular and Organismal Defense Mechanisms

The immune system is the basic system of the body that defends the organism against foreign invaders such as viruses and bacteria. This system is important in maintaining the health of the organism and involves multiple cell types as well as two main branches: the innate immune system and the adaptive immune system.

Adaptive Immune System

The adaptive immune system is the more complex and specific form of the immune system. This is because B and T lymphocytes are produced in this system to actively fight off and kill pathogens. B and T cells are two forms of white blood cells that can provide antibody responses as well as cell-mediated responses (Alberts et al., 2002). Upon activation, B cells develop into plasma cells, which actively secrete antibodies, or immunoglobulins, and memory B cells, which provide an effective response to additional infections of the same pathogen. Antibodies target and bind to antigens of the pathogen and mark the pathogen for destruction. In the cell-mediated response, T cells react to surface antigens of pathogens and either directly kill them or help other cells destroy them (Alberts et al., 2002).

T cells include helper T cells (T_H) as well as cytotoxic T cells (T_C). Both cell types have T cell receptors that are highly variable in order to recognize antigens of pathogens. They do not, however, bind the antigen directly. Instead, they utilize antigen presenting cells (APCs) through major histocompatibility complexes (MHCs). The APCs include dendritic cells and macrophages, which are both a part of the innate immune systems. The antigen of the pathogen is presented to the T cell through the MHC. T_H cells recognize MHC class II through CD4 receptors; T_C cells recognize MHC class I molecules through their CD8 receptors.

Innate Immune System

The innate immune system is the first line of defense because it is immediately activated upon being introduced to a pathogen. This system uses nonspecific defense mechanisms: antiviral, antibacterial, and inflammatory responses. Innate immune cells such as dendritic cells, macrophages, and neutrophils recognize pathogen-associated molecular patterns (PAMPs) through their pattern recognition receptors (PRRs) (Kumar, Kawai, & Akira, 2011). One example of a PAMP is lipopolysaccharide (LPS). LPS is also known as an endotoxin and can go through autolysis to initiate immune responses (Freudenberg et al., 2008). Because it is common to all gram-negative bacteria, it is easily recognizable through PRRs. Examples of PRRs include toll-like receptors (TLRs) and retinoic acid–inducible gene I like receptors (Kawai & Akira, 2009). When PRRs bind PAMPs, a signaling pathway is initiated that results in the activation of the immune system. This occurs because the pathway leads to transcription factor activation to transcribe functional genes of the immune system. Common transcription factors include nuclear factor-κB (NF-κB) and interferon (IFN) regulatory factors, which regulate the expression of cytokines, chemokines and inflammatory factors that facilitate the removal of the pathogen (Kumar, Kawai, & Akira, 2011). The innate immune system also helps to activate the adaptive immune system through initiating the production of specific B and T lymphocytes.

TLRs are the most understood PRRs and are heavily involved in sensing pathogens (Kumar et al., 2011). TLR4 is the PRR responsible for detecting LPS when complexed with the protein MD2 (Kumar et al., 2011; Hegazy et al., 2015). After binding, the TLRs form homodimers, leading to a series of intracellular signaling involving NF-κB (Liang et al., 2004). The result of the pathway is the production of proinflammatory cytokines such as tumor necrosis

factor alpha (TNFα) and interlukein-6 (IL-6). In a majority of the innate immune responses, convergence at NF-κB occurs because it is the key transcription factor in transcribing Type I IFNs and inflammatory cytokines. When NF-κB is inactive, it remains in the cytoplasm of the cell bound to inhibitors of NF-κB (IκBs) (Liang et al., 2004). To activate the transcription factor, the PRR binding results in phosphorylation of the IκB kinase (IKK) which, in turn, phosphorylates the IκB. The phosphorylation stimulates the degradation of the IκB (Liang et al., 2004). The NF-κB dimer previously attached is released, freeing up the nuclear localization signal. The dimer is then translocated into the nucleus, where it interacts with DNA to express Type I IFNs and inflammatory cytokines (Liang et al., 2004).

Innate Immunity in Differentiated Cells and ESCs

The innate immune system has been intensively studied in differentiated somatic cells; however, we have limited knowledge about the innate immunity of ESCs and ESC-differentiated cells. Recent studies have shown that both ESCs and ESC-differentiated cells lack a fully functional innate immune system. For example, ESC-differentiated cells have limited responses to various infectious agents and inflammatory cytokines (Guo et al., 2015). This includes ESC-derived endothelial cells, cardiomyocytes and smooth muscle cells from both human and mouse ESCs (D'Angelo et al., 2016). On the other hand, endothelial cells, cardiomyocytes and smooth muscle cells differentiated naturally are sensitive to the same infectious agents (D'Angelo et al., 2016). However, innate immune cells such as dendritic cells and macrophages derived from ESCs or iPSCs are able to perform their immune functions (Guo et al., 2015).

ESCs also have an underdeveloped innate immune system. ESCs are susceptible to bacterial and viral infection because hESCs and mESCs both do not express MHCs or express them in low levels (Drukker et al., 2002). In addition, the IFN system, essential to a cell's innate

immunity, is not fully functional in ESCs (D'Angelo et al., 2016). In particular, ESCs are unable to express type I IFNs in response to viral stimuli as well as having attenuated responses to these cytokines (D'Angelo et al., 2016). Type I IFNs, including IFNα and IFNβ, are proteins that are secreted by infected cells; they function to activate the innate immune system (Ivashkiv & Donlin, 2014). It was determined that ESCs are unable to express IFNα; they also have a limited responsiveness to the cytokines TNFα and IFNγ (D'Angelo et al., 2016). In addition, ESCs were resistant to treatment with LPS. The reason ESCs do not produce or respond to type I IFNs, viral and bacterial stimuli is due to the NF-κB pathway not being activated (D'Angelo et al., 2016).

Upon differentiation, the NF-κB pathway becomes activated, allowing the cells to respond to immune and inflammatory stimuli. This is evident in the fact that embryonic stem cell-differentiated fibroblasts (ESC-FBs) are sensitive to the same cytokines that caused little to no response in ESCs (D'Angelo et al., 2016). Based on the fact that ESCs lack effective responses to various cytokines and infectious agents, it is clear that the innate immune system is not "innate" in ESCs, but it is rather acquired upon differentiation.

Inflammatory Cytokine-Induced Oxidative Stress

TNFα and IFNγ as Embryotoxic Cytokines

TNFα and IFNγ are two cytokines that are considered embryotoxic due to their involvement with impaired embryo development and implantation failure. TNFα, while involved in multiple cellular events, has been identified in causing embryonic death through triggering immunological pregnancy loss (Toder et al., 2003). Elevated TNFα levels during implantation has been involved in induction of apoptosis (Toder et al., 2003). Controlled apoptosis is needed during development to have organogenesis occur correctly through sculpting structures and

eliminating harmful cells (Toder et al., 2003). When levels of TNFα increase uncontrollably, developmental abnormalities or spontaneous abortion can occur. However, in order to prevent the birth of offspring with grave structural abnormalities, TNFα, in the appropriate levels, is essential in initiating apoptotic signaling cascades (Toder et al., 2003).

IFNγ works in similar ways to initiate apoptosis during embryogenesis. IFNγ is the only member of the type II interferons group (Kotredes & Gamero, 2013). During early pregnancy, it is secreted by uterine natural killer cells (Murphy et al., 2009). Upon secretion, IFNγ's role is to allow successful implantation through initiating endometrial vasculature remodeling and angiogenesis of the implantation site (Murphy et al., 2009). If this process is uncontrolled and levels of IFNγ secretion are changed, apoptosis may occur resulting in the potential of unsuccessful implantation or impaired embryo development. Largely, IFNγ is known to activate the innate and adaptive immune systems through upregulating transcription of genes involved in apoptosis and cell cycle regulation (Murphy et al., 2009). When IFNγ is used in combination with TNFα, apoptosis is initiated (Kotredes & Gamero, 2013). Due to the maternal reproductive tract secreting both TNFα and IFNγ during the implantation process, the environment could be detrimental to the embryo if they are excessively produced (Robertson et al., 2018). TNFα and IFNγ levels are also elevated during inflammatory conditions such as infection, which is known to both help fight off infection and cause collateral damage to the normal cells. The two cytokines synergistically cause damage by inducing apoptosis through different mechanisms. The induction of inducible NOS (iNOS), which produces cytotoxic nitric oxide (NO), is one of the mechanisms underlying the cytotoxicity of TNFα and IFNγ in mouse cells.

Induction of iNOS and Production of NO

Nitric oxide (NO) is an important regulatory molecule at low levels in many cellular processes, but it can act as a free radical product that causes cytotoxicity depending on the enzymatic source and the amount produced (Thannickal & Fanburg, 2000). NO is produced in multiple tissue types and has various functions including vasodilation, smooth muscle relaxation, stimulation of the immune response and embryo implantation (Gouge et al., 1998). NO is produced by the enzyme nitric oxide synthase (NOS). There are three isoforms of NOS: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS) (Gouge et al., 1998). Each isoform is located in different tissues with varying functions, as seen in Figure 3. nNOS functions in both the central nervous system and peripheral nervous system, to control blood flow, synaptic plasticity, and vasodilation (Förstermann & Sessa, 2012). eNOS is located mostly in the endothelium: the inner epithelial lining of blood vessels, the heart and lymphatic tissues. It functions in controlling blood pressure. Lastly, iNOS is expressed in many cell types, in response to inflammatory stimuli, such as LPS, TNFα and IFNγ (Förstermann & Sessa, 2012).

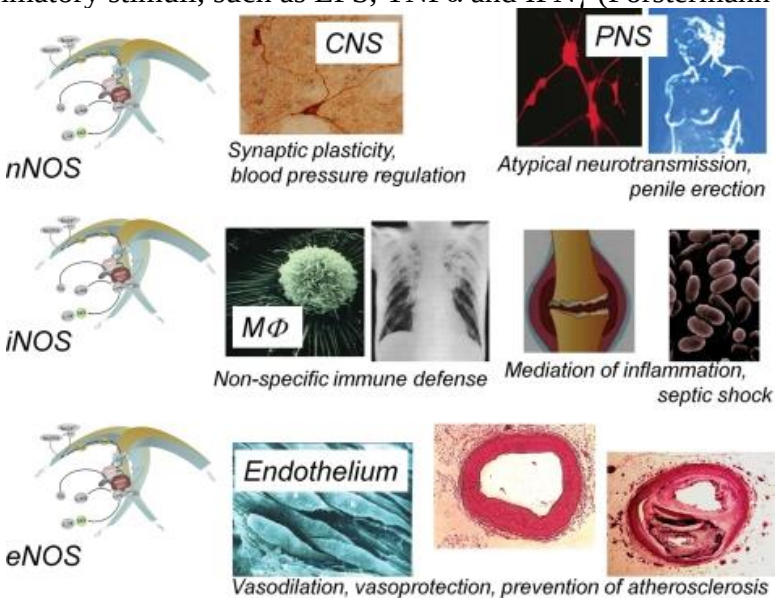

Figure 3: Functions of the Various Isoforms of NOS (image obtained from Förstermann & Sessa, 2012)

In eNOS and nNOS, lower concentrations of NO are produced to perform their functions;

however, in iNOS, high levels of NO are produced to function in microbial killing (Thannickal & Fanburg, 2000). These high concentrations, while effective in killing microbes, can also cause damage to normal cells and tissues. This process has been linked to high levels of inflammation and septic shock, a dangerous condition where too much fluid leaves the blood system that can result in death (Förstermann & Sessa, 2012).

The iNOS pathway is induced by paracrine and autocrine signaling of IFNγ and TNFα. IFNγ and TNFα are both produced in response to external stimuli binding receptors on the cell membrane (Samuel, 2001). The binding results in a signaling cascade, causing the expression of the iNOS gene (Figure 4). Upon binding, primarily, the initiation of the Janus tyrosine kinase (JAK) and signal transducers and activators of transcription (STATs) (JAK-STAT) pathway occurs (Samuel, 2001). JAK1 and JAK2 phosphorylate the intracellular domain of the receptor (Samuel, 2001). The phosphorylation provides a docking site for STAT1 which is subsequently phosphorylated (Samuel, 2001). STAT1 translocates to the nucleus where it acts as a transcription factor for iNOS proteins among other genes of the immune response (Samuel,

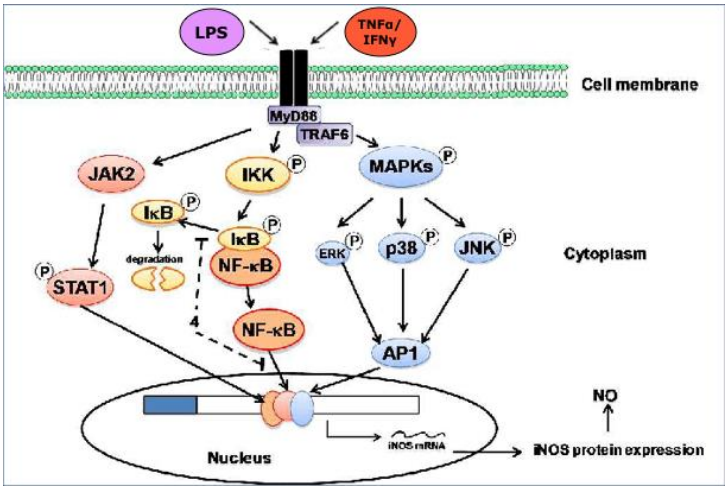

Figure 4: Mechanisms of the Induction of iNOS to Produce NO (image modified from Hegazy et al., 2015)

2001). If other cytokines or PAMPs bind, the NF-κB pathway and the mitogen-activated protein

kinases pathway may be initiated instead (Hegazy et al., 2015). All of these converge with transcription factors affecting the transcription of the iNOS gene. When the mRNA is translated and iNOS is expressed, high levels of NO is produced.

Nitric Oxide Function in Embryogenesis and Oxidative Stress

NO has also been linked to important physiological events during the developmental process. NO levels are regulated by estrogen during the implantation process and may help the process be successful (Gouge et al., 1998). In the endometrium, dramatic changes occur to allow for implantation of the embryo including proliferation and increased vasculature. Both changes are similar to inflammatory processes (Chwalisz & Garfield, 2000). While the implantation process is not completely understood, it is known that NO is involved. In mouse endometrium, iNOS and eNOS are upregulated at the site of implantation (Chwalisz & Garfield, 2000). NO is also upregulated in the placenta where blood vasculature must be formed to provide nutrients for the growing embryo (Chwalisz & Garfield, 2000).

In the preimplantation embryo, NO acts through the cyclic guanosine 3′,5′-monophosphate (cGMP) pathway (Tranguch et al., 2003). NO and cGMP are necessary in a narrow window of concentrations for the development of the embryo, and if it deviates, apoptosis of the embryo can occur (Tranguch et al., 2003). Treating an embryo with high concentrations of SNP, a NO donor, caused the arrest of the embryo (Tranguch et al., 2003). In addition, NO inhibitors resulted in the embryo arresting, so it is believed that NO in low levels or high levels can cause problems in embryogenesis (Tranguch et al., 2003). All isoforms of NOS were tested to determine which played roles in NO production in the early embryo at different stages of development. All three isoforms were expressed except iNOS in early blastocysts

(Tranguch et al., 2003). It is important to mention that it is from the early blastocyst that ESCs are derived.

In addition, NO functions in oxidative stress. When NO production is unregulated, cellular damage can occur. Oxidative stress, in general, is the result of heightened levels of reactive oxygen species (ROS) outweighing the production of antioxidants (Erusalimsky, 2020). Oxidative stress, which can result from normal metabolic activity or disease states, can affect many cellular activities (Burton & Jauniaux, 2011). Because ROS is produced as a byproduct of normal metabolism, cells have mechanisms to detoxify molecules that produce ROS. For example, peroxisomes are involved in detoxifying hydrogen peroxide. If the levels of ROS exceeds that of the antioxidant activity, oxidative stress can occur (Thannickal & Fanburg, 2000). If the levels of ROS are too high, cells may go through apoptosis or enter a senescent phase if the levels are sublethal (Song et al., 2005). In cellular senescence, cells no longer have the ability to divide. Other than causing cellular damage, ROS are also considered to be cellular signals. A balance between ROS as important modulators of metabolic activity and ROS serving as toxic by-products must be maintained. It is believed that this balance comes from the ROS concentration (Thannickal & Fanburg, 2000). This is especially seen in NO, as explained previously.

CHAPTER TWO: RATIONALE, HYPOTHESIS, OBJECTIVES

The early embryo, before implantation, is at a very vulnerable stage of development where it faces various inflammatory cytokines during the implantation process. In this stage, the blastocyst must still proliferate in spite of its environment. Previous studies have shown that ESCs do not respond to TNFα and IFNγ: two inflammatory cytokines that induce strong inflammatory responses in most differentiated cells. Treatment of mESC-differentiated fibroblasts (mESC-FBs) with TNFα and IFNγ in combination (TNFα/IFNγ) significantly reduced cell viability and the rate of cell proliferation; however, this treatment has no effect on the cell viability and the cell cycle of mESCs. It has been previously demonstrated that inducible nitric oxide synthase (iNOS) stimulated by TNFα/IFNγ is responsible for the effects of TNFα/IFNγ, since NO produced by iNOS is a free radical that can cause cellular damage. Based on this finding, it is hypothesized that the resistance of mESCs to TNFα/IFNγ cytotoxicity is due to mESCs lacking a response to these two cytokines. Therefore, iNOS and NO were not produced, allowing mESCs to avoid the cytotoxicity of TNFα/IFNγ. To test this hypothesis, this project used sodium nitroprusside (SNP), a NO donor, to determine the sensitivity of mESCs to NO. It is expected that SNP treatment would result in decreased cell viability through increasing apoptosis, which would suggest that mESCs are susceptible to the cytotoxicity caused by NO. Therefore, the lack of iNOS induction by TNFα/IFNγ in mESCs may help to protect mESCs from the cytotoxicity of the two cytokines at the early stage of embryogenesis.

This project has great significance because it will lead to a better understanding of the immune properties of ESCs in their pluripotent stage and their differentiated cells. The molecular mechanisms are important in determining the biological implications of underdeveloped innate immunity in ESCs, in particular their lack of responses to TNFα/IFNγ.

CHAPTER THREE: EXPERIMENTAL DESIGN AND METHODS

Cell Culture

mESCs (D3 cell line, ATCC) and mESC-FBs were used throughout the project. For mESCs, Dulbecco's modified Eagle medium (DMEM), supplemented with 15% fetal bovine serum (FBS) and 1000 U/mL of leukemia inhibitory factor (LIF, a cytokine that maintains the stem cell state of ESCs), was utilized. For mESC-FBs, DMEM plus 10% FBS was used without LIF. Cells were cultured at 37 °C in a humidified incubator with 5% CO_2, as previously described (Wang et al., 2014)

When the cells reached 70-80 percent confluence, they were subcultured in new dishes. First, medium, phosphate-buffered saline (PBS) and trypsin were heated to 37 ºC in a water bath. The old medium in the dish was removed and cells were washed with PBS. 1 mL of trypsin was added to the culture (6-well dish) to detach the cells, and the cells were incubated for 2 minutes. The dish was tapped gently to dislodge the cells. After the cells were detached, they were collected into a 2 mL tube and 1 mL of medium was added to inactivate the trypsin. The cells were then centrifuged at 1,200 rpm for 3.5 minutes. The supernatant was carefully removed, without disrupting the cell pellet. The pellet was resuspended in 1 mL of fresh medium and then replated on a new dish at 30-50% confluence.

Cells were treated 24 hours after they were seeded. The same steps were followed when passaging cells except the cells were plated in wells of a 12, 24, or 48-well plate depending on the experimental conditions. For mESCs, a confluence of 40-60% was used for effective treatment to occur. Waiting 24 hours past seeding ensures that the cells have attached to the surface of the plate. At this point, the volume of SNP needed to have a 50 μM, 100 μM or 150 μM treatment concentration, again depending on the experimental conditions, was calculated.

The SNP was added directly to the medium. The plate was then incubated for the treatment time according to the experimental conditions, either 24 or 48 hours.

Cell Viability Analysis

Toluidine Blue (TB) staining is used to indirectly determine cell viability. The cells were fixed with cold methanol and stained with 1% TB staining solution (150 µl per well for 48 well plate) for 60 minutes. The TB was removed using a bulb pipette carefully to prevent the cells from being dislodged from the bottom of the well. The wells were then washed thoroughly with water until it ran clear, leaving only the cells stained blue. Sodium dodecyl sulfate (SDS) acts as a detergent and extracts the stain from the cells. 150 µl of 2% SDS was added to each well. After the TB in the cells was completely extracted into the solution, the absorbance at 630 nm was determined using a microtiter plate reader. The value obtained correlated with the number of viable cells determining cell proliferation and cell viability indirectly. Blanks were also used by staining the bottom of empty wells in order to account for the staining of the plate.

DAPI Staining

DAPI, 4′,6-diamidino-2-phenylindole, is a blue fluorescent stain that binds the AT-rich regions of double stranded DNA. It was used to stain the nucleus, which contains DNA, blue in order to analyze for nuclear fragmentation. 1 µL of 10 µg/mL DAPI was added to 500 µL of PBS and added to each well containing a coverslip for an hour. It was kept in a dark environment to protect the fluorescence. After staining, the coverslips were then mounted onto a microscope slide with 20 µl of mounting medium. First, excess liquid was removed from the coverslip by touching the edges of it to a paper towel. The coverslip was then transferred to the mounting

medium cell-side down. Excess mounting medium was removed using a paper towel. The microscope slides with attached coverslips were covered and placed in 4 ºC for 1 hour. After this, two coats of clear nail polish were brushed on around the edges of the coverslip to seal the coverslip to the slide. The cells were viewed using a Nikon ECLIPSE 80i Fluorescence Microscope, and the images were photographed using a digital camera.

Gene Expression Analysis by Polymerase Chain Reaction (PCR)

RNA Extraction

The cells were collected using 0.5 mL of TRIzol per well of a 6 well dish. TRIzol is used because it homogenizes the tissues while keeping the integrity of RNA, DNA and proteins. The cells were collected and pipetted into 1.5 mL tubes. The sample stood for 5 minutes then 100 μL of chloroform was added. The sample was vortexed for 15 seconds and was left to stand at room temperature for 10 minutes to initiate phase separation. It was then centrifuged at 10,000 rpm for 10 minutes at 4 °C. The aqueous phase was then transferred into another tube ensuring that the interphase was not disturbed during transfer. The aqueous phase contains the RNA while the interphase contains DNA. The organic phase contains protein. 250 μL of isopropanol and 1 μL of glycogen were added per tube. Isopropanol and glycogen help to precipitate the RNA out of the solution to form a white pellet. The sample was then incubated in -20 °C for at least 1 hour to allow the RNA to precipitate. After the incubation, the sample was centrifuged at 12,000 rpm at 4 °C for 15 minutes. The supernatant was discarded watching carefully to ensure the pellet does not get disturbed. 800 μL of 75% ethanol was added and vortexed until the pellet was dislodged. The sample was then centrifuged at 10,000 rpm at 4 °C for 10 minutes. Carefully, the ethanol was poured out and any remaining ethanol was removed with a pipette. The RNA was left to air

dry on ice for 5 minutes. At this point, the pellet was no longer visible and it was resuspended with DEPC water, 10-20 μL depending on the size of the pellet with less water for smaller pellets. The concentration and integrity of the RNA was checked using a spectrophotometer to determine the ratio of A_{260}/A_{280} (the value should be within the range of 1.8-2.2). The RNA was then stored at -70 °C and used for complementary DNA (cDNA) synthesis.

Reverse Transcription (cDNA synthesis)

cDNA must be synthesized from the RNA previously extracted in order to quantify it during PCR amplification. In order to do this, 1 μg RNA in DEPC water was added to get a total volume of 6 μL. 0.5 μL of dNTP mix (10 μM) and 1 μL of oligo (dT) primer (10X) was added to the sample. The sample was then incubated at 70 °C for 5 minutes in a thermal cycler and then placed on ice. After incubation, 0.5 μL of M-MLV reverse transcriptase and 2 μL of 5X Moloney Murine Leukemia Virus (M-MLV) buffer (provided by the manufacture, Promega) were added to initiate reverse transcription reaction. The reaction mix was incubated in the thermal cycler at 42 °C for 1 hour, then 10 minutes at 95 °C to inactivate the enzymes. Lastly, 90 μL of DEPC water was added and it was stored at -20 °C until needed.

Quantitative Real-Time PCR (RT-qPCR)

For RT-qPCR analysis, a master mix was made for each treatment group being tested in 0.5 mL tubes. For the master mix, the following was added per well: 5 μL of 2X SYBR mix, 1.5 μL of DEPC water and 2.5 μL of cDNA template, made during cDNA synthesis. 10% extra of each were added as well to account for pipetting errors. 1 μL of primer, specific for the genes of interest, was added to each PCR tube. Next, 9 μL of the master mix was added. PCR tubes were then put into a Strategene MX3000P real-time PCR thermal cycler. The samples were run for 35 cycles (5 min at 95°C for initial denaturation, then 35 cycles of 95°C for 15s for denaturation

plus 60°C for 1 min for annealing/elongation), and the data was collected using MxPro software. The data was then analyzed using Ct values relative to β-actin expression. β-actin serves as a housekeeping as previously described (Wang et al., 2014). The data was analyzed using the comparative Ct method:

$$\frac{expression\ of\ experimentalgroup}{expression\ of\ control\ group} = \frac{2^{(C_T\ \beta actin - C_T\ gene\ of\ interest)\ experimental}}{2^{(C_T\ \beta actin - C_T\ gene\ of\ interest)\ control}}$$

The following primers were used:

Gene	Forward Primer Sequence	Reverse Primer Sequence
β-actin	CATGTACGTAGCCATCCAGGC	CTCTTTGATGTCACGCACGAT
Oct4	AGTTGGCGTGGAGACTTTGC	CAGGGCTTTCATGTCCTGG
Sox2	GACAGCTACGCGCACATGA	GGTGCATCGGTTGCATCTG
Nanog	TTGCTTACAAGGGTCTGCTACT	ACTGGTAGAAGAATCAGGGTC
Cyclin A2	ACATTCACACGTACCTTAGGGA	CATAGCAGCCGTGCCTACA
Cyclin D1	CAGAAGTGCGAAGAGGAGGTC	TCATCTTAGAGGCCACGAACAT
Cyclin E1	CCTCCAAAGTTGCACCAGTTTGC	GACACACTTCTCTATGTCGCACC
Cdk2	CTCGACACTGAGACTGAAGGT	GCAGCTTGACGATATTAGGGTGA
p19	ATGCTGGATTGCAGAGCAGTA	ACGGGGCACATTATTTTTAGTCT
p21	CGAGAACGGTGGAACTTTGAC	CAGGGCTCAGGTAGACCTTG

Oct4, Sox2, and Nanog were used to test the relative expression of pluripotency markers to determine stem cell state. Cyclin A2, Cyclin D1, Cyclin E1 and Cdk2 primers were used to test cell cycle changes. p19 and p21 primers were used to test expression levels of cell cycle inhibitors.

CHAPTER FOUR: RESULTS

Characterization of mESCs

To determine any effects of cell treatments, the morphology of normal mESCs was examined under a phase contrast microscope. As shown in Figure 5, mESCs exhibit typical ESC morphology of undifferentiated cells where the cells grow together in clustered colonies. They also have a large nucleus-to-cytoplasmic ratio. The image shows a colony of mESCs that was photographed with a digital camera 3 days after growth in cell culture dish.

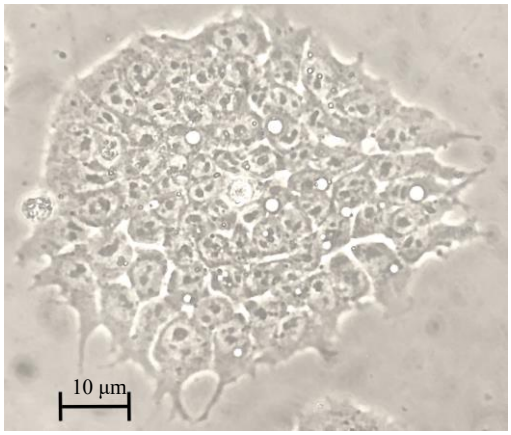

Figure 5: Morphology of mESCs. mESCs were seeded on cell culture dish and allowed 3 days to proliferate. mESCs examined and imaged under phase contrast microscope at 200X plus 2X zoom on camera (Scale bar unit = 10 μm).

mESCs and mESC-FBs are Sensitive to SNP Cytotoxicity

Because NO is known as a ROS, a series of experiments was performed to determine the effect of SNP treatment, a NO donor that chemically produces NO in cell culture. It was expected that treatment with SNP would cause decreased cell viability and increased levels of apoptosis in both mESCs and mESC-FBs, due to there being more ROS in culture. The mESCs and mESC-FBs were seeded on a 48-well culture plate at a confluence of 40-50% and 50-60% confluence, respectively. They were allowed one day for attachment and then treated with various concentrations of SNP for 24 or 48 hours and fixed. The absorbance at 630 nm, which correlates with the number of attached cells, was measured with a plate reader after TB staining.

As seen in Figure 6A, the numbers of cells in the cell culture for both cell types decreased significantly with increased concentrations of SNP treatment. This is indicated by the smaller colony size in mESCs and low cell density in mESC-FBs. Furthermore, there are increased numbers of dead cells in mESC-FBs.

In order to quantitatively determine the cell number, the cells stained with TB in the culture dishes were extracted with 2% SDS solution. The absorbance of SDS solution that contains TB, which is indirectly their relative cell number in each experimental condition, was determined. As see in Figure 6B, SNP treatment resulted in decreased cell number in both mESCs and mESC-FBs. SNP at 150 μM reduced the number of viable cells by ~70% and ~80% at 24 and 48 hour treatment, respectively. It was noticed that the effect of SNP on mESCs was inversely related to cell density. Although mESC-FBs are more tolerant that mESCs to the cytotoxicity of SNP, SNP at 150 μM treatment for 48 hours caused about 55% decrease of viable cells. These results clearly demonstrated that both mESCs and mESC-FBs are susceptible to the cytotoxicity of SNP in a dose-dependent manner.

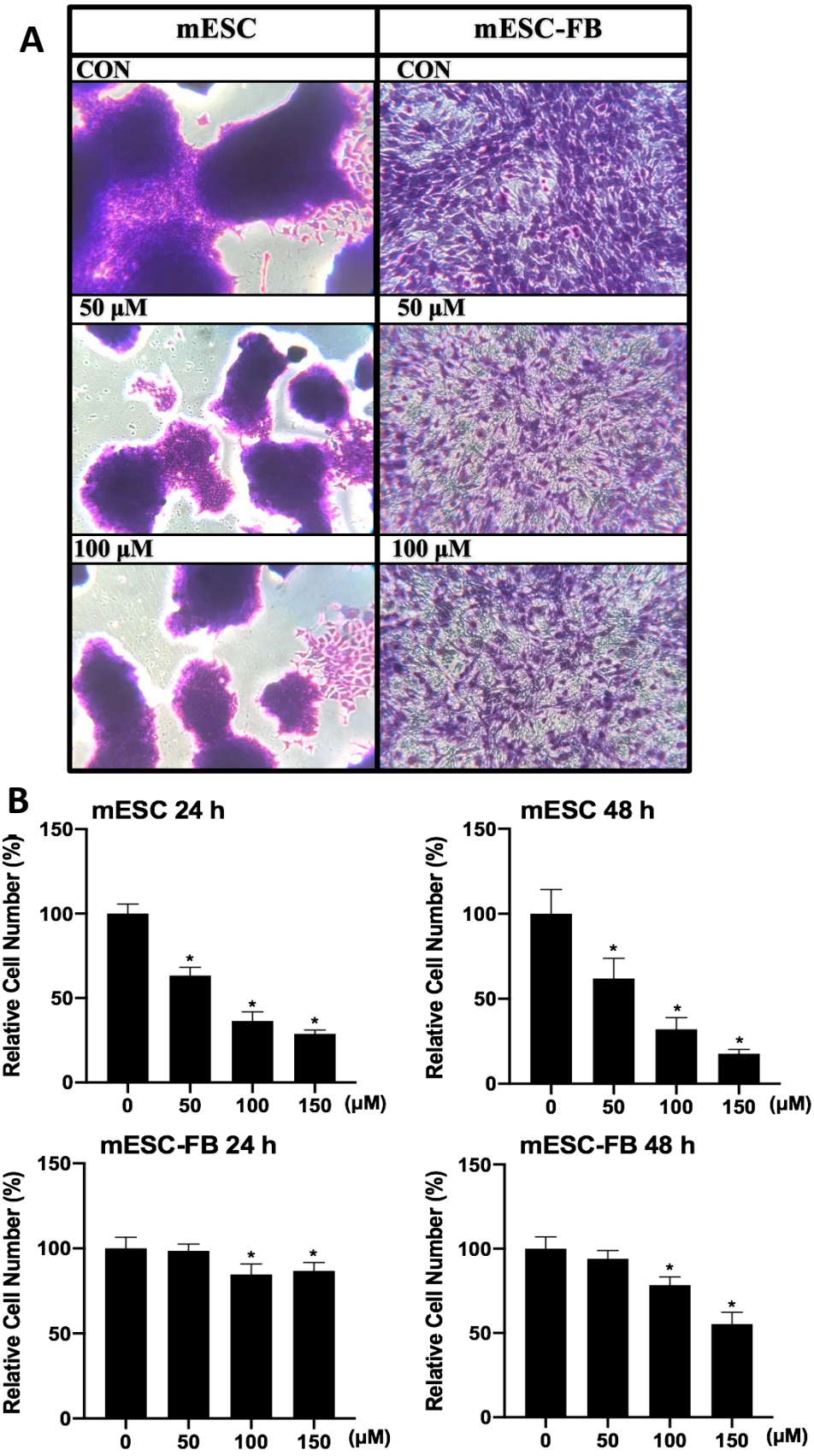

Figure 5: Effects of various concentrations of SNP on the viability of mESCs and mESC-FBs. (A) The morphology and cell density of mESCs and mESC-FBs after TB staining. mESCs and mESC-FBs were seeded on 48-well culture plate. After incubation for 24 hours to allow cells to attach, cells were exposed to SNP for 24 hours then fixed. TB stained cells were examined and photographed with a light microscope at 100X. (B) Cell viability analysis by TB staining. The cell number in the control experiment, 0 μM (control), was taken at 100%. The results are means ± SD from a representative experiment carried out in triplicate. The experiment was performed at least 3 times with similar results. Statistical analysis was completed using a 2-tailed unpaired Student's *t*-test. Differences are considered statistically significant when **P*< 0.05 compared with the value of the control group.

SNP Causes Apoptosis of mESCs

Oxidative stress can cause cytotoxicity in different forms, including induction of apoptosis, inhibition of cell proliferation, or loss of cell specific function. The previous experiments clearly demonstrated that SNP can inhibit cell proliferation of mESCs, as indicated by the reduced colony size (Figure 6A). To determine if SNP can cause apoptosis, mESCs were seeded onto coverslips at a confluence of 30%. They were allowed one day for attachment and then underwent 150 μM SNP treatment. The cells in the control experiment were not treated with SNP. After 24 hours, the cells were fixed with methanol and stained with DAPI. The coverslips were then mounted to a microscope slide and examined under a fluorescence microscope.

In the control, the cells grew in compact colonies as seen in Figure 7A. Individual cells can be detected by their round blue nuclei stained with DAPI. The cells treated with 150 μM SNP lost normal colony morphology. In addition to dramatically reduced colony size, the nuclei on many cells are fragmented, which is a major characteristic of apoptotic cells (Figure 7B, in the inset and indicated by arrows).

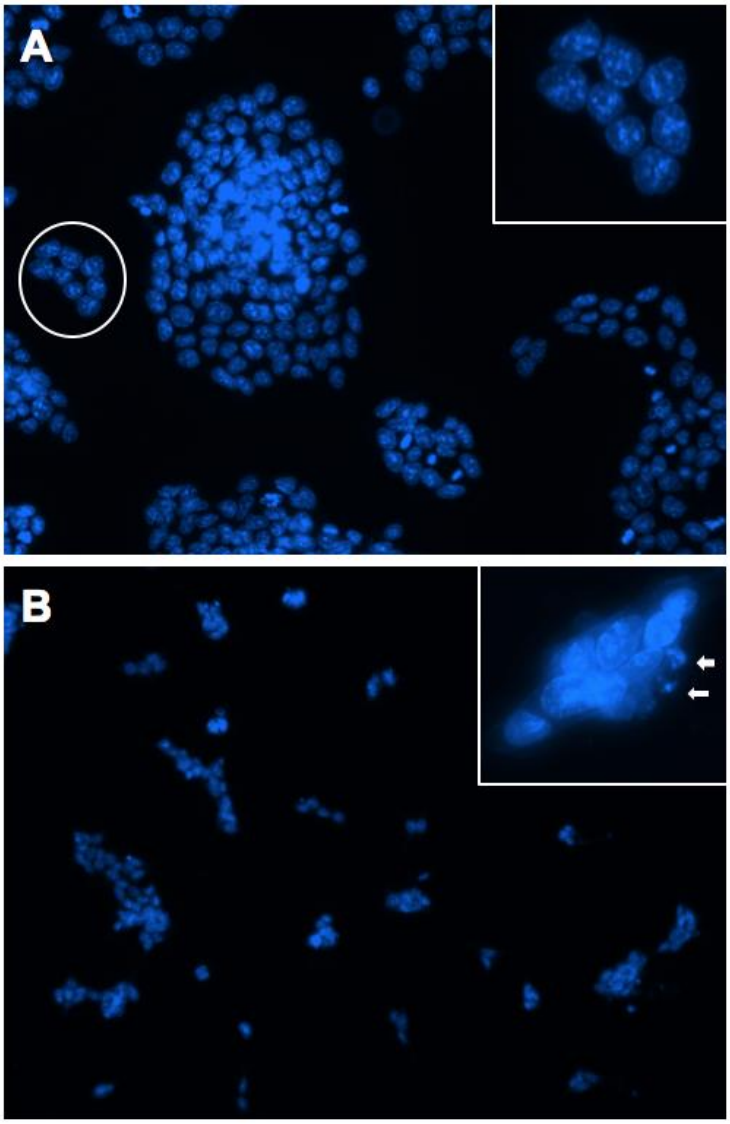

Figure 7: SNP-induced apoptosis in mESCs. Control mESCs (A) or cells treated with 150 μM SNP for 24 hours (B) were fixed then stained with DAPI. The cells were examined and photographed using Nikon ECLIPSE 80i Fluorescence Microscope at 200X. Insets represent an enlarged area (circled) (400x) that shows intact nuclei (A) and apoptotic cells (B, fragmented nuclei were indicated by arrows).

The Effect of SNP on mESC Pluripotency

The stem cell properties of mESCs are maintained by the expression of ESC specific genes that include Oct4, Sox2, and Nanog (Schnerch et al., 2010). To test if the expression of these genes were affected by SNP treatment, the expression of the above-mentioned genes was analyzed by RT-qPCR. The mRNA was isolated from control mESCs and cells treated with 150

μM SNP for 48 hours were used to determine the expression levels of Oct4, Sox2 and Nanog compared to that of the control. As shown in Figure 8, ESCs exposed to SNP showed a significant decrease in Nanog expression with a 50% decrease in comparison with control cells (CON), whereas the expression levels of Oct4 and Sox2 were not significantly changed.

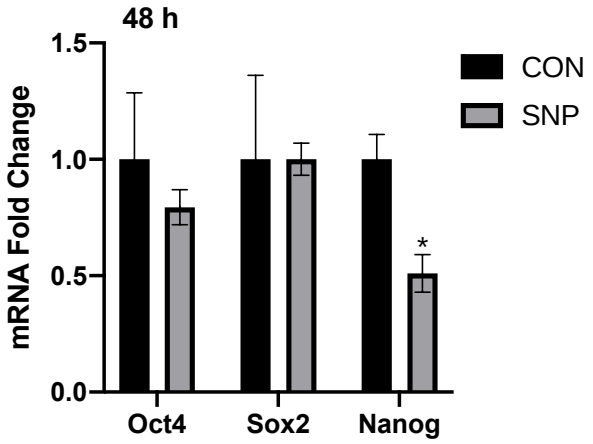

Figure 8: Effect of SNP on the expression of mESC pluripotency markers. ESCs were treated with 150 μM SNP for 48 hours. The mRNA levels of the tested genes were analyzed by RT-qPCR and expressed by fold change. The mRNA in the control cells are designated as 1. The values are means ± SD of a representative experiment performed in triplicate. The experiment was conducted three times with similar results. Statistical analysis was performed using a student's *t*-test with differences considered significant when $*P<0.05$.

The Effect of SNP on the Expression of Cell Cycle Regulators in mESCs

Effect of SNP on mESC Cyclins and Cyclin-Dependent Kinase (CDK)

In addition to pluripotency, the question of whether SNP treatment resulted in a significant change in the cell cycle of mESCs was studied. mESCs have a unique cell cycle in comparison to that of somatic cells. They have shorter gap phases and a large portion of the cells are in the S phase of the mitotic cycle. The S phase is where DNA synthesis takes place to replicate the genome (White & Dalton, 2005). Cell cycle progression is mainly regulated by cyclins and CDKs. Cyclin E1 and CDK2 work together to help initiate DNA replication and

progression through the G1-phase of the cell cycle (Mazumder et al., 2004). In addition, Cyclin D1 participates at the G1-phase of the cell cycle by activating CDK4 and CDK6 (Liu et al., 2017). Cyclin D1 and Cyclin E1 are both considered to be G1 cyclins, and this group, with their CDKs, is needed to stabilize the pluripotency factors Oct4, Sox2 and Nanog (Liu et al., 2017). Cyclin A2 works in a different manner in the cell cycle. It serves to activate DNA replication in the S phase, in addition to activating the G2 to M phase transition (Kalaszczynska et al., 2009). Cyclin A2, like Cyclin E1, works with CDK2 to function properly (Kalaszczynska et al., 2009). To investigate the molecular mechanism that led to SNP-induced reduced cell number, the expression of several cell cycle regulators was examined. RT-qPCR analysis was performed on mESCs that underwent 150 μM SNP treatment for 48 hours. The relative mRNA expression levels of cell cycle regulators, including Cyclin A2, Cyclin D1, Cyclin E1, CDK2, were determined. As seen in Figure 9, Cyclin A2, Cyclin D1, Cyclin E1 and CDK2 have no significant changes in mRNA expression for 48 hour treatment in comparison with control cells.

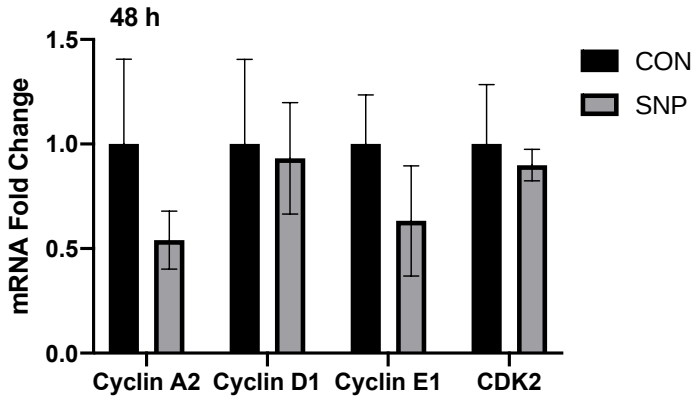

Figure 9: The Effect of SNP on the expression of mESC Cyclins and Cyclin-Dependent Kinase. mESCs were treated with 150 μM SNP for 48 hours then collected. The mRNA levels of the tested genes were analyzed by RT-qPCR and expressed by fold change. The mRNA in the control cells are designated as 1. The values are means ± SD of a representative experiment performed in triplicate. The experiment was conducted three times with similar results. Statistical analysis was performed using a student's *t*-test, but there were no significant differences between control and treated groups.

Effect of SNP on mESC Cell Cycle Inhibitors

In addition to cyclins and CDKs as activators of the cell cycle, the cell cycle is also negatively regulated by inhibitors. Cell cycle inhibitors slow or stop the progression of the cell cycle through various mechanisms and can act at differet parts of the cell cycle. p19 and p21 are two cell cycle inhibitors that inactivate CDKs, so with increased levels, they prevent the proliferation of cells (Capparelli et al., 2012). The upregulation of p19 and p21 expression is also associated with the onset of senescence (Ben-Porath & Weinberg, 2005). As seen in Figure 10, p19 and p21 had no significant changes in mRNA expression in mESCs treated with 150 μM SNP for 48 hours in comparison with control cells. Overall, SNP treatment caused no significant changes to cell cycle markers even though apoptosis was inniated.

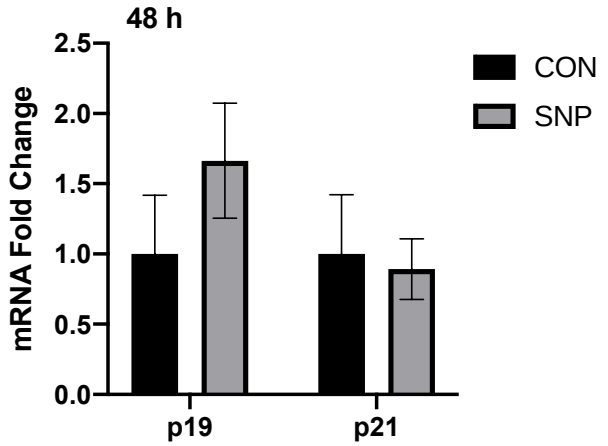

Figure 10: Effect of SNP on mESC Cell Cycle Inhibitors. mESCs were treated with 150 μM SNP for 48 hours then collected for RT-qPCR. The mRNA levels of the tested genes were analyzed by RT-qPCR and expressed by fold change. The mRNA in the control cells are designated as 1. The values are means ± SD of a representative experiment performed in triplicate. The experiment was conducted three times with similar results. Statistical analysis was performed using a student's *t*-test with no significant differences between control and treated groups

CHAPTER FIVE: DISCUSSION

We have limited knowledge about immunoproperties of ESCs, but it is known that they have underdeveloped innate immunity and lack of responses to a wide range of infectious agents that induce strong immune and inflammatory reactions in differentiated cells. The biological implications of this finding are poorly understood at the present time and more studies will be needed. This study sought to understand one aspect of the attenuated immune response in ESCs: the molecular mechansims underlying the resistance to the cytotoxicity of the cytokines TNFα and IFNγ. Although these two inflammatory cytokines are widely involved in most types of immune and inflammatory responses in mammals, they play specific roles during early embryogenesis, in particular, the implantation process of the blastocyst. Since TNFα and IFNγ synergistically induce apoptosis in many mouse somatic cells, including ESC-FBs, through the induction of the iNOS pathway, the insensitivity of mESCs to these two cytokines becomes particularly interesting. A previous study has provided strong evidence that this insensitivity is due to the signaling pathways that mediate the effects of TNFα and IFNγ are not functional (D'Angelo et al., 2018). Because the signaling pathway is not functional, it explains the finding that mESCs do not activate the iNOS pathway in response to TNFα/IFNγ treatment while differentiated cells do (D'Angelo et al., 2018). Without iNOS induction in mESCs, they would not have a chance to be exposed to NO, which could otherwise be cytotoxic. However, this finding does not answer the question of whether mESCs are susceptible to NO. The results provided an answer to this question and demonstrates that mESCs are indeed susceptible to NO.

In this study, the effects of SNP treatment on mESCs were examined at multiple levels. The first aspect examined was the effect of SNP on cell viability for both mESCs and mESC-FBs. mESC-FBs were used as a positive control system since they are known to acquire the

ability to express type I IFNs and respond to TNFα upon differentiation (Wang et al., 2014; D'Angelo et al., 2016), and importantly, they are sensitive the cytotoxicity of TNFα/IFNγ (D'Angelo et al., 2018). The results of this study clearly demonstrated that both mESCs and mESC-FBs are susceptible to SNP cytotoxicity since mESCs treated with SNP showed nuclear fragmentation, the typical feature of apoptotic cell death. Together with decreased cell density and disrupted colonies, these results serve as indicators that mESCs were sensitive to SNP due to the production of NO in culture.

While the results clearly indicate that apoptosis is a major form of SNP-induced cytotoxicity in mESCs, there were no significant changes in the expression of pluripotency marker genes except Nanog in SNP treated mESCs. Likewise, the mRNA levels of cell cycle inhibitors, p16 and p19, were not affected. In addition, p19 and p21 are known to be associated with the onset of senescene in somatic cells through their upregulation by preventing the proliferation of cells (Ben-Porath & Weinberg, 2005; Capparelli et al., 2012). Although SNP treatment resulted in reduced mRNA levels of cyclins and CDKs to different levels, the changes were not statistically significant. These findings suggested that the two major characteristics, pluripotency and high rate of proliferation, appeared not significantly impacted by SNP under the experimental conditions described in this study (150 μM SNP for 48 hours). However, it should be pointed out that the effects of SNP on expression of the above-mentioned molecules at the protein level were not tested. As a result, at this time, it is uncertain how the stem cell properties are effected by SNP treatment. More detailed studies under more defined experimental conditions, such as longer exposure times of mESCs to SNP at sub-lethal dosages for several passages, will be necessary to determine the long-term effects. More rigorous analysis of cell cycle by flow cytometry and gene expression analysis by Western blotting will be needed. In

addition, to confirm the results obtained from SNP, a molecular biology approach can be used by expressing iNOS gene through the use of a plasmid in mESCs. It is expected that iNOS expressed from the plasmid could bypass the need of TNFα/IFNγ stimulation to induce iNOS; therefore, the result would give direct proof as to the function of the iNOS pathway in causing cytotoxicity.

Although more research needs to be conducted in order to understand the exact mechanisms by which mESCs can avoid the cytotoxicity of TNFα/IFNγ, it seems that the lack of iNOS expression at the blastocyst stage of development plays a key role in preventing the oxidative stress associated with NO. This makes physiological sense, since mESCs are indeed sensitive to the cytotoxicity of NO, as we demonstrated in this study. Therefore, the lack of iNOS induction by TNFα/IFNγ in mESCs may serve as a protective mechanism to avoid the cytotoxicity of the two cytokines during the inflammatory processes of early embryogenesis. While this study focused on a small aspect of mESC biology, the finding makes a meaningful contribution in understanding how mESCs avoid potential damages caused by immunologic cytotoxicity that the blastocyst may encounter during the process of implantation.

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