2010

Cell extract-based reprogramming of somatic cells

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CELL EXTRACT-BASED REPROGRAMMING OF SOMATIC CELLS

**A Thesis
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science**

in

**The Interdepartmental Program of
Animal and Dairy Sciences**

by

Laura Whitney Coley

B.S., Louisiana State University, 2007

December 2010

ACKNOWLEDGEMENTS

The author would like to thank her fellow graduate students both past and present for their encouragement, help, and advice throughout this time of learning. A very special thank you is extended to Alicia Picou for mentoring the author during her first year of graduate school. The author would also like thank Cody Bailey for all of his "technical" support with the final preparations of this thesis project. Lastly, the author would like to thank Meredith Addison for all of her contributions to this research project.

Of course, none of this would be possible without the love and support of the author's parents, Howard and Ann Coley. Throughout the author's life, they have encouraged her to do nothing but her best. Their constant support, encouragement, love and motivation instilled within the author the desire to pursue a higher education. Sincerest gratitude and love are owed to the author's loving family.

Last, but not least, the author would like to express her appreciation and gratitude to Dustin Gaspard for all he has done to aid in the completion of this research project. Throughout the author's Master's program, she often joked how she would be completely lost without him, as it was Dustin who calmed her before a big test and brought her dinner when he knew she had not taken a break from her studies to eat. All joking aside, without you, my life would be incomplete.

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LIST OF ABBREVIATIONS

ADAS……………………………………………….......Adipose-derived Adult Stem Cells
ASC……………………………………………….………Adult Stem Cells
ATP…………………………………………………..……… Adenosine 5'-triphosphate
BCP……………………………………………………..1-bromo-3 chloropropane
BFF……………………………………………………..Bovine Fetal Fibroblast
CS .... Calf Serum
C_T .... Threshold Cycle
C_TR... Threshold Cycle for the Reference Gene
C_TT .... Threshold Cycle for the Target Gene
DEPC……………………………………………………….Diethyl pyrocarbonate
DMEM………………………………………………Dulbecco's Modified Eagle Medium
DTT……………………………………………………………….Dithioreitol
DPBS .... Dulbecco's Phosphate-Buffered Saline
ESC…………………………………………………..Embryonic Stem Cells
FBS .... Fetal Bovine Serum
GTP……………………………………………………..… Guanosine 5'-triphosphate
hESC…………………………………………………..Human Embryonic Stem Cells
hASC…………………………………………………..Human Adult Stem Cells
HMG…………………………………………………………High Mobility Group
HSC…………………………………………………..Hematopoietic Stem Cells
ICM... Inner Cell Mass
iPSC…………………………………………………..Induced Pluripotent Stem Cells
MEF…………………………………………….……………Mouse Embryonic Fibroblast
MSC…………………………………………………..Mesenchymal Stem Cells
PAP .... Poly(A) Polymerase
PI .... Propidium Iodide
P/S .... Penicillin/Streptomycin
PSOS……………………………………………………Porcine Skin Oriented Sphere
qRT-PCR....................Quantitative Reverse Transcriptase Polymerase Chain Reaction
RT .... Reverse Transcription
RT-PCR .... Reverse Transcriptase Polymerase Chain Reaction
SCNT .... Somatic Cell Nuclear Transfer
SLO…………………………………………………………Streptolysin O

ABSTRACT

The differentiation potential of adult stem cells (ASC) has long been thought to be limited to cell lineages present in the organ from which they are derived; however, several studies have challenged this notion by demonstrating that some ASC exhibit a remarkably high degree of plasticity. Unlike terminally differentiated somatic cells, the less differentiated state of ASC can assume the functional phenotypes and expression profiles of cells unique to other tissues. The expansive repertoire of differentiation potential exhibited by ASC suggests these cells possess characteristics similar to pluripotent cells, including epigenomic regulatory pattern.   Therefore, ASC may be better equipped for complete epigenetic reprogramming than terminally differentiated cells.

The objective of Experiment 1 was to analyze bovine adipose-derived adult stem cells (ADAS) and fetal fibroblast (BFF) cells for the presence of the pluripotency-associated genes, Oct-4, Nanog, and Sox-2. Because the endogenous expression of these genes is believed to contribute to reprogramming efficiency, Experiment 2 sought to increase Oct-4, Nanog and Sox-2 expression levels in BFF cells through exposure to ADAS cell extracts.

Transcripts for all three pluripotency-associated genes were detected in all BFF and ADAS cell samples at every passage analyzed; however, expression was quite low and highly variable between cell lines and passage numbers.   Nevertheless, these findings support the notion that these cells are less differentiated than other somatic cells. This less differentiated state appears to sufficient for at least the partial

reprogramming of BFF cells using ADAS cell extracts in a cell extract-based nuclear

reprogramming system.

CHAPTER I

INTRODUCTION

The extraordinary nature of embryonic stem cells (ESC) lends them the remarkable ability to give rise to all the cell types of a mammalian organism, an attribute known as pluripotency. The pluripotent state of ESC is contingent upon the expression of the genes Oct-4, Nanog and Sox-2, which have also been identified as the key transcriptional regulators of pluripotency (Boyer et al., 2005). Until recently, Oct-4, Nanog and Sox-2 were believed to be expressed solely in ESC; however, studies have reported the expression of these genes in some sources of adult stem cells (ASC) of both the mouse and pig (Kues et al., 2005; Carlin et al., 2006). Furthermore, these ASC as well as several other sources of ASC have been shown to exhibit a surprisingly high degree of plasticity. This remarkable feature allows ASC to cross lineage barriers and adopt the expression profiles and functional phenotypes of cells unique to other tissues (Herzog et al., 2003). These findings suggest that ASC possess characteristics similar to pluripotent ESC, including an epigenetic regulatory pattern that may be more amenable to reprogramming than that of other somatic cells. Considering the abundance and accessibility of adipose tissue, adipose-derived adult stem cells (ADAS) cells are an attractive source of stem cells for use in research and biomedical applications.

In the present study, we first examined cells derived from bovine adipose tissue as well as fetal fibroblasts (BFF) for the expression of Oct-4, Nanog and Sox-2. It has been suggested that the genome of less differentiated cells may be more amenable to

reprogramming or require less reprogramming following the induction of pluripotency either by somatic cell nuclear transfer (SCNT) or another experimental strategy (Rideout et al., 2001). Because of the high degree of plasticity demonstrated by ADAS cells, it is likely that these cells have an epigenomic regulatory pattern that is closer to pluripotent ESC than terminally differentiated somatic cells (Sun et al., 2009).

Because the endogenous expression of Oct-4, Nanog and Sox-2 is believed to contribute to reprogramming efficiency, we next attempted to increase the expression levels of these genes by exposing BFF cells to ADAS cell extracts. Extract-based reprogramming approaches have shown that differentiated cells may be induced to transdifferentiate into other differentiated cell types (Collas and Håkelien, 2003) or dedifferentiate towards pluripotency (Taranger et al., 2005; Bru et al., 2008). Because the genome of ASC is inherently less differentiated than other somatic cells, exposing them to extracts of other ASC may drive them further towards an undifferentiated state. Extract-based reprogramming using ASC is a promising and plausible approach towards the production of replacement cells for therapeutic purposes, as well as, the production of suitable donor cells for SCNT.

CHAPTER II

LITERATURE REVIEW

Stem Cell Basics

Stem cells are unlike any cell type, and, of the many different cell types known, none are able to mimic the phenomena of stem cells. ESC possess the ability to become any cell type, and, as a result, possess the ability to revolutionize the scientific and medical community. The most popular and perhaps the greatest application of stem cells are in regenerative medicine. Regenerative medicine is designed to replace or restore cells, organs and tissues that have been damaged or destroyed by disease (Hipp and Atala, 2008). Interest in regenerative medicine has heightened throughout the years as the demand for organs and tissues in order to satisfy the ever-growing number of individuals awaiting transplant far exceeds the supply. Stem cells have the ability to create an abundance of cells and tissues to replace those that are damaged or injured. In addition, stem cells promise treatment of diseases such as Parkinson's and Alzheimer's diseases, as well as healing of spinal cord injury, stroke, burns, heart disease, diabetes, osteoarthritis and rheumatoid arthritis. Yet stem cells' potentials are not limited to medical applications. Studies of stem cells allow scientists to gain a greater understanding of the molecular and genetic controls responsible for the development of specialized cells from stem cells. Better understandings of the mechanisms regulating this process permit scientists to study malfunctions that can occur during this development (Odorico et al., 2001). Consequently, stem cells warrant

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a great deal of attention, not only from the scientific community, but also from the general public.

Embryonic Stem Cells

From the first few days of gestation through adulthood, stem cells are present in all mammalian species. Throughout the life cycle, however, stem cells change. The stem cells present in the embryo shortly after fertilization are known as embryonic stem cells (ESC) (Evans and Kaufman, 1981; Martin, 1981)whereas the stem cells present in an adult are known as adult stem cells (ASC). In the mouse, nonhuman primates, and humans, ESC are derived from the inner cell mass (ICM) of a blastocyst-stage embryo. As gastrulation progresses, the stem cells of the ICM will differentiate into the primitive ectoderm, which ultimately gives rise to the three germ layers: ectoderm, endoderm, and mesoderm. Under the appropriate culture conditions, these cells can proliferate rapidly and, theoretically, remain in an undifferentiated state, capable of generating any cell type of the three germ layers, indefinitely (Odorico et al., 2001).

Adult Stem Cells

Unlike ESC which are detected only in the ICM of a blastocyst, ASC are found in a variety of adult mammalian tissues and organs. ASC primarily function to replenish damaged cells within these tissues and organs as a result of normal cellular senescence or injury (Odorico et al., 2001). Researchers have identified many sources of ASC, including skin, blood and the brain, although, the most utilized source for these cells is bone marrow (Ratajczak et al., 2007). Although interest in ASC has dramatically increased in recent years, their potential in cell-based therapies has long been

established. Since the first procedure in 1956, bone marrow transplants have been used to treat patients with certain cancers, such as leukemia and lymphoma, as well as individuals suffering from illnesses like Sickle cell anemia and Thalassemia. Subsequently, a great deal of research in ASC has focused on these cells, known as hematopoietic stem cells (HSC). Within the bone marrow of an adult animal, HSC function to replenish the damaged or dead cells of all the hematopoietic lineages throughout its lifetime (Goodell et al., 1996). HSC function similarly in bone marrow transplants, repopulating the reserve of bone marrow and generating healthy new blood cells (Hipp and Atala, 2008). However, the success of bone marrow transplants are limited by the number of available donors, which need to have the same human leukocyte antigens as the recipient in order to prevent graft rejection (Cairo and Wagner, 1997). Despite this limitation, bone marrow transplants have proven to be successful in the treatment of several life-threatening illnesses for over 50 years. The identification of HSC in bone marrow prompted researchers to explore other sources as potential reservoirs for HSC, which resulted in the discovery of these cells in placental and umbilical cord blood (Broxmeyer, 1989). Collectively referred to as cord blood, this relatively small quantity of blood contains a significantly higher concentration of HSC than adult bone marrow and also exceeds the culture life span of adult bone marrow (Hows et al., 1992). Perhaps the greatest attribute of cord blood is that it is far less immunogenic than adult peripheral blood, thus enabling its successful application in hematopoietic stem cell transplant for both related and unrelated donors (Cairo and Wagner, 1997).

Years after the discovery of HSC in bone marrow, researchers identified a non-hematopoietic population of ASC in the stromal fraction of bone marrow, now known as mesenchymal stem cells (MSC). MSC are often referred to as multipotent adult progenitor cells because of their ability to differentiate into many cell lineages, including osteoblasts, adipocytes, chondrocytes, myoblasts, and early progenitors of neural cells (Sekiya et al., 2002). This intrinsic property of MSC suggests enormous potential in cell-based therapies, fueling research since their original discovery in bone marrow stromal cells (Strem et al., 2005).   MSC have since been isolated from a variety of tissues, including adipose tissue, which provides a potentially abundant and easily accessible source of ASC (Zuk et al., 2001).

Unique Properties of Stem Cells

While the source and primary function of stem cells differ between these two cell types, both ESC and ASC possess the definite and unique characteristics specific to stem cells. It is these distinctive characteristics that make them especially valuable in clinical application. One unique aspect of stem cells is their remarkable potential to develop into many different cell types. When a stem cell divides, it can either become another stem cell or it can become another type of cell with a more specialized function. The ability of stem cells to take on the form of different cell types is called differentiation (Odorico et al., 2001). ESC are capable of forming all cell types, but most ASC can only differentiate into the cell type of the tissue from which they are found. This is why ASC are often referred to as tissue-specific cells.   However, there are certain kinds of ASC that are capable of differentiating into cells of a variety of tissue.   For example, bone marrow is one of the most common sources of ASC. This is because it is a source of

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different types of stem cells, therefore, capable of developing into a variety of differentiated cell types.   Specifically, bone marrow stromal cells are capable of generating cells that can develop into bone, cartilage, fat, and fibrous connective tissue (Sekiya et al., 2002).

Unlike ASC, ESC are identified in only one location, and their differentiation potential is not limited in regards to tissue type. Instead, ESC are pluripotent. A pluripotent stem cell is able to develop into any type of cell of the endoderm, ectoderm, or mesoderm, but it cannot differentiate into embryonic membranes. Because ESC cannot give rise to extra-embryonic membranes, they are not totipotent and labeling them as such is incorrect.   Alternatively, the zygote is totipotent, and it remains totipotent through the morula stage. Following the morula stage, cells enter the blastocyst stage, and it is at this time that the trophoblast cells develop and migrate to the periphery of the cell to form what will be the placenta. Encompassed inside the trophoblast is the inner cell mass that will form the fetus. Cells of the inner cell mass are pluripotent, and these are the cells used for research and medical applications. The ability of stem cells to undergo differentiation is one of the characteristics that make them unlike any other cell type, therefore, capturing the interests of many.

Stem cells are able to differentiate into various cell types because they are unspecialized. Stem cells, unlike other cells in the body, do not perform a specialized function because they lack any tissue-specific structures. Despite ASC being found with other tissue-specific cells, they are less differentiated than the surrounding somatic cells, and retain the ability to renew and differentiate to yield the major specialized cell types of the tissue (Sekiya et al., 2002). Because stem cells are unspecialized they

cannot carry out the critical functions of heart cells or neural cells; however, these unspecialized stem cells can give rise to specialized cells like heart cells and neural cells. It is this feature that gives stem cells their great potential.

In addition to the properties mentioned, stem cells are also characterized by having the ability to divide and renew themselves for extended periods of time. As previously discussed, stem cells can differentiate into a number of other cell types, yet they can also replicate many times to create more stem cells, remaining unspecialized. If the cells continue to replicate for an extended period of time, it is known as proliferation.   Proliferation of a stem cell line in a laboratory setting for a few months can result in millions of stem cells (Odorico et al., 2001), thus, ensuring their ability of long-term renewal.

The Problem with Stem Cells

Despite the promise of stem cells, there are many obstacles to overcome before they can fully express their potential in medical applications.   Since its emergence, immune rejection has plagued transplant and gene therapy, limiting its success in some patients. Immune rejection is also a potential complication of stem cell therapy, particularly with ESC, and, while immunosuppressive drugs limit the possibility of rejection, their use has been associated with numerous complications, including wound healing, opportunistic infections, drug-related toxicities, skin malignancies, and post-transplant lymphoproliferative disorders (Odorico et al., 2001).

Furthermore, undifferentiated stem cells have been shown to produce tumors in animals, reinforcing this as a potential consequence of stem cell therapy (Donovan and

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Gearhart, 2001).   Technical hurdles, such as the establishment of optimal ESC culture conditions are another obstacle yet to be overcome. For ESC to be an effective tool in regenerative medicine, they must be produced in large quantities and be able to differentiate into a homologous cell population (Donovan and Gearhart, 2001). While promising advances in stem cell research frequently emerge, not all of stem cells' impediments can be resolved by science. Unfortunately, the remarkable potentials of stem cells are often overshadowed by the controversy surrounding them, particularly ESC. All ESC are truly pluripotent, exceeding the inherent differentiation capabilities of ASC, but, because ESC are derived from the inner cell mass of a blastocyst, there are ethical concerns over the use and subsequent disposal of human embryos. In fact, government funding of ESC research has been limited for the past 8 years due to these moral concerns. However, ESC research regained momentum on March 9, 2009, when President Obama removed these limitations in the hopes of potentiating the capabilities of stem cells in medical applications. While this barrier has been removed, research in ASC and stem cell alternatives have not been neglected and continue to be frontrunners in scientific research.

ASC are present in many organs and tissues, including mesenchyme, adipose tissue, and endothelial tissue (Gimble et al., 2007), therefore they are an attractive and successful option for the treatment of many diseases. Hematopoietic stem cell transplants have been successfully used to treat many patients with life-threatening diseases, such as leukemia for years. From 1988 to 1997, there were an estimated 500 related and unrelated hematopoietic stem cell transplants using umbilical cord blood (Cairo and Wagner, 1997). ASC have had an unprecedented impact on cell-based

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therapies; however, despite the proven capabilities of ASC in regenerative medicine, ASC are multipotent, which limits their differentiation capabilities and, ultimately, their therapy potentials to their tissue of origin. The promise of a treatment for diseases such Type I diabetes, in which pancreatic islet cells have been destroyed, or Parkinson's disease, which results from the destruction of dopaminergic neurons truly lies within the use of ESC (Odorico et al., 2001). In fact, insulin- producing pancreatic islet-like cells have been generated from ESC, (Murtaugh and Melton, 2003), and studies in the rat have proven that dopamine neurons, derived from ESC, develop functional synapses and display electrophysiologic properties characteristic of midbrain neurons following transplantation, providing a realistic approach to the treatment of Parkinson's disease (Kim et al., 2002).

In addition, there are typically only a small number of stem cells within an organ or tissue, and research has shown that it is difficult to produce large numbers of these cells once in culture because ASC lose their proliferative and differentiation capabilities as cell cultures are expanded (Sekiya et al., 2002), further limiting their use in medicine. Undoubtedly, ASC have made immense contributions to the success of cell- based therapy, proving to be effective in the treatment of several diseases; however, the multipotent nature of ASC limits the variety of cell types that can be produced, rendering them inherently incapable of producing the certain cell types needed for the treatment of other diseases.

Pluripotency in Embryonic Stem Cells

The ability of ESC to basically become any cell type is the foundation of their potentials, verifying pluripotency as their most notable and heralded property. Despite the early stage of development and relatively small number of cells, the cells of the ICM express their pluripotent potential by giving rise to the 3 germ layers: ectoderm, endoderm and mesoderm, which will eventually give rise to all the organs and tissues of the body (Odorico et al., 2001). The proper development and progression of the preimplantation embryo to the implanting blastocyst is dependent upon carefully regulated molecular and cellular events within the embryo (Ovitt and Schöler, 1998)

Proper development of the early stage embryo relies upon a precise series of events controlled by the expression of specific genes. Conversely, accurate gene expression is dependent upon transcriptional activity, which is responsible for regulating biological processes throughout life. In recent years, interest and subsequent research in stem cells has escalated, resulting in several advances in stem cell biology. Many of these advances are due to the identification and characterization of ESC transcription factors, especially those responsible for regulating pluripotency. Researchers have identified Oct-4, Sox-2, Nanog, Klf4 and Myc as the key pluripotency-regulating transcription factors in ESC (Takahashi and Yamanaka, 2006).

Oct-4 Expression in Embryonic Stem Cells

Octamer 4, designated as Oct-4, is a homeodomain transcription factor of the POU family essential for the expression of pluripotency in ESC (Ovitt and Schöler, 1998). Oct-4 is found not only in ESC, but in primordial germ cells (PGC) and

unfertilized oocytes (Schöler et al., 1990), as well as embryonal carcinoma cells (Ovitt and Schöler, 1998). Originally, Oct-4 is present as maternal RNA and protein in unfertilized oocytes. As the case with most maternal RNAs, however, maternal Oct-4 RNA and protein levels decrease upon fertilization, rendering it undetectable past the 2-cell stage of embryogenesis. It is at this time, the 4 to 8 cell stage, that zygotic Oct-4 gene expression is activated in the mouse embryo (Schöler et al., 1990). All blastomeres at this early-cleavage stage express Oct-4 uniformly; however, the expression of Oct-4 becomes restricted as development progresses. Those cells of the morula destined to form the placenta, migrate to the periphery of the developing embryo and differentiate into the cells of the trophectoderm (Kehler et al., 2004). Consequently, Oct-4 is down-regulated in these peripheral cells and is expressed solely by the cells of the ICM. As embryonic development continues, Oct-4 is restricted once again as the cells of the ICM differentiate into the hypoblast and epiblast. The expression of Oct-4 is maintained in the cells of the epiblast, which are responsible for the formation of the 3 germ layers during gastrulation; however, Oct-4 expression is undetectable in the cells of the hypoblast as they differentiate (Kehler et al., 2004). During gastrulation, Oct-4 expression in the epiblast is gradually reduced until it is confined solely to primordial germ cells (PGC) (Schöler, 1990). As the preimplantation embryo progresses through the stages of embryogenesis, cells differentiate, and, as a result, Oct-4 expression is limited to the decreasing number of undifferentiated cells.

Nanog Expression in Embryonic Stem Cells

At each ESC division, cell fate is determined by the expression, or lack thereof, of regulatory intrinsic factors (Chambers et al., 2003). The fate of the cells of the preimplantion embryo is first determined at the morula stage, where cells that retain the expression of Oct-4 are sequestered to the ICM, and those that do not express Oct-4 differentiate into the trophectoderm (Kehler et al., 2004). After the formation of the blastocyst, the cells of the ICM rapidly proliferate, selectively differentiate and reorganize as a pluripotent cell population under the direction of another transcription factor, known as Nanog (Mitsui et al., 2003). Like Oct-4, Nanog is a homeobox-containing transcription factor that plays an essential role in regulating early embryonic development and maintaining pluripotency in the cells of the ICM (Chambers et al., 2003). Both Oct-4 and Nanog function in the same restricted expression mechanism to specify pluripotency in ESC, but their roles in this process are unique (Boyer et al., 2005). Nanog expression levels are highest between the late morula and mid blastocyst stage of embryogenesis, when the pluripotent cells of the ICM divide a second time into the epiblast and hypoblast. A pluripotent cell population is maintained in the epiblast by restricting Nanog expression to these cells (Chambers et al., 2003). Disruption of this expressive pattern causes ESC to improperly differentiate into hypoblast, and deletion of the Nanog gene curtails pluripotency in both the ICM and ESC (Chambers et al., 2003; Mitsui et al., 2003). Therefore, Nanog plays an indispensible role in early embryonic development and in the maintenance of pluripotency in ESC.

Sox-2 Expression in Embryonic Stem Cells

In addition to Oct-4 and Nanog, Sox-2 has also been identified to play an essential role in early embryonic development and the propagation of pluripotency in ESC. Sox-2 is a member of the Sex Determining Region-Y (SRY)-High Mobility Group (HMG) box gene family that acts to maintain the developmental potential of ESC (Avilion et al., 2003). Sox-2 is not only expressed in the ICM, epiblast, and germ cells, but it is also present in multipotent cells of the extraembryonic ectoderm (Avilion et al., 2003). In the mouse embryo, Sox-2 RNA is distinctively present in the ICM of the blastocyst, although some cells of the morula stage embryo express Sox-2. As Nanog directs the division of the ICM into the epiblast and hypoblast, Sox-2 is confined to the epiblast, where it continues to be expressed until the mid-late streak stage of development. At this time, Sox-2 expression is confined to the anterior portion of the primitive streak, specifically the presumptive neuroectoderm, however, it is the expression of Sox-2 at a second location that results in its presence in the extraembryonic ectoderm. The extraembryonic ectoderm forms from the polar trophectoderm, the portion of the trophectoderm in direct contact with the ICM, and gives rise to the various cell types of the placenta and other structures such as the chorion. By day 9.5 postcoitum, Sox-2 is present throughout the mouse brain, neural tube, sensory placodes, branchial arches, gut endoderm, and both male and female germ cells (Avilion et al., 2003). Furthermore, ESC that are Sox-2 deficient improperly differentiate into trophectoderm-like cells, confirming that like Oct-4 and Nanog, Sox-2 is an indispensible factor in the maintenance of ESC pluripotency (Masui et al., 2007).

Transcriptional Regulation of Oct-4, Nanog and Sox-2 in Embryonic Stem Cells

Oct-4, Nanog and Sox-2 are the key components of the transcriptional regulatory circuitry that governs pluripotency in ESC. This intricate circuitry involves the up and down regulation of Oct-4, Nanog, and Sox-2 target genes, which ensure proper early embryonic development and the reproduction of undifferentiated ESC. The transcriptional regulation circuitry responsible for ESC identity is dependent upon the precise action of a series of complex molecular and cellular mechanisms. The exact mechanism responsible for regulating ESC self-renewal and pluripotency is still largely unknown, perhaps because the function of the molecular and cellular machinery responsible for these properties is not fully understood (Loh et al., 2006). Nevertheless, a great deal of research has recently focused on providing a greater understanding of the mechanisms involved in maintaining the properties of ESC.

In 1981, Evans and Kaufman demonstrated that mouse ESC could be expanded and maintained in culture by co-culturing them with mitotically inactive fibroblast cells; however, it was not until 1988 that the factor responsible for promoting self-renewal while inhibiting differentiation was identified (Smith et al., 1988; Williams et al., 1988). Leukemia Inhibitory Factor (LIF), a member of the IL-6 cytokine family, promotes pluripotency in ESC by activating STAT3. LIF mediates the activation of STAT3 through a receptor complex composed of a low affinity LIF receptor, known as LIFRβ, and gp130. The binding of LIF to the LIFRβ-gp130 heterodimer activates STAT3 in ESC through a process of tyrosine phosphorylation, dimerisation and translocation of the dimerized STAT3 signaling molecule to the cell nucleus, which initiates the transcription

of STAT3. Activation of this transcription factor is absolutely essential for the self-renewal of mouse ESC (Matsuda et al., 1999); however, neither LIF nor STAT3 is directly responsible for maintaining the pluripotent state of ESC (Mitsui et al., 2003). Instead, the LIF receptor, gp130, mediates the activation of STAT3, which regulates gene expression by interacting with Oct-4. Similar cell differentiation patterns result when either Oct-4 is overexpressed or STAT3 is inactivated, illustrating an intersection between these two transcription factors (Mitsui et al., 2003).   Alternatively, Nanog is capable of sustaining self-renewal of ESC in the absence of STAT3 activation; therefore, its function is independent of LIF/STAT3 pathway (Chambers et al., 2003). Chambers et al (2003) also showed that an overexpression of Nanog is capable of promoting ESC self-renewal in the absence of STAT3 activation, but maximum self-renewal efficiency requires both Nanog and STAT3 activity. Interestingly, loss-of-function experiments resulted in the differentiation of similar cell types, suggesting that although there is no direct link between these factors, Nanog and STAT3 function in a corresponding manner to potentiate ESC's character (Chambers et al., 2003).

Indeed, none of these factors can maintain ESC identity alone. Therefore, Oct-4, Nanog and Sox-2 rely upon one another for this intrinsic circuitry to properly function as a whole. At the 4-cell stage of embryonic development, the regulatory circuitry of ESC identity is activated by the ubiquitous expression of Oct-4 (Ovitt and Schöler, 1998). A cell-restrictive change in this expression pattern marks the developing embryo's first cell differentiation event, subsequently forming the trophectoderm and the pluripotent ICM. Oct-4 maintains the undifferentiated state of the ICM by acting as both a transcriptional repressor and activator (Ben-Shushan et al., 1998). As a member of the POU domain

family of octamer-binding proteins, Oct-4 activates or represses its target genes by

binding to their specific octamer site (Ovitt and Schöler, 1998). By silencing the

transcriptional activity of appropriate genes, Oct-4 binding represses the untimely

expression of two genes, whose expression is later required for embryo implantation

and pregnancy maintenance. Alternatively, the binding of Oct-4 to pluripotency-

associated genes, including the Oct-4 gene itself, activates the transcription, and

subsequent expression, of these developmentally important genes (Ovitt and Schöler,

1998).

Oct-4 potentiates its functional activity in ESC through interactions with other

transcription factors, such as Sox-2 (Loh et al., 2006).   A member of the HMG-domain

DNA-binding protein family, Sox-2 is absolutely required to maintain ESC identity. In

contrast to the regulatory mechanisms utilized by Oct-4 and Nanog, Sox-2 indirectly

maintains pluripotency by regulating transcriptional activity of Oct-4 (Masui et al., 2007).

By maintaining the requisite levels of Oct-4 expression, Sox-2 is able to reciprocally

function with Oct-4 in the direct regulation of ESC pluripotency. In fact, the

transcriptional activity of the pluripotency-associated genes, *Fgf4, Utf1, and Fbx15* is

regulated by the collaborative action of Oct-4 and Sox-2 (Yuan et al., 1995; Nishimoto et

al., 1999; Tokuzawa et al., 2003). These genes each contain an Oct-4-specific binding

site and a Sox-2-specific binding site, and, while these two factors bind independently,

their interaction is essential for proper early embryonic development (Masui et al.,

2007). When this dual binding occurs, Sox-2 forms a ternary complex with Oct-4 protein

in the enhancer element of the target gene, and, therefore, regulates its expression as a

binary complex, known as Oct-4/Sox-2 complex (Loh et al., 2006).   In addition to the

aforementioned genes, the regulatory region of both Sox-2 and Pou5f1, the gene encoding Oct-4, contain binding sites for the Oct-4/Sox-2 complex; therefore, these enhancer regions are the direct targets of their own gene products (Chew et al., 2005). The Oct-4/Sox-2 complex secured its position at the top of the pluripotent regulatory network when researchers found the Nanog proximal promoter also contains an Oct-Sox element that must be bound by Oct-4 and Sox-2 for Nanog transcription (Kuroda et al., 2005; Rodda et al., 2005). Furthermore, the regulatory elements of each of the aforementioned genes contain the Oct-4 site and Sox-2 site within 3-bp of each other, which further elucidates a cooperative role in the regulation of pluripotency (Chew et al., 2005).

Recently Boyer et al (2005) have shown that Oct-4, Nanog and Sox-2 co-occupy regulatory regions of at least 353 genes and two miRNA genes in human ESC. Indeed, prior studies have explored the relationship between these three transcription factors which suggested a collaborative effort in the regulation of pluripotency (Kuroda et al., 2005; Rodda et al., 2005), and yet Boyer et al (2005) were admittedly surprised to discover the multitude of genes containing binding sites for Oct-4, Nanog and Sox-2. In addition to their mutual target genes, all three transcription factors contribute to ESC pluripotency and self-renewal by also activating their own genes (Boyer et al., 2005).

The defining properties of ESC are dependent upon the precise execution of a network of activated and repressed genes, regulated in tandem by the transcriptional activity of this hierarchical threesome. These transcription factors work synergistically by forming a tight and complex intrinsic regulatory circuit that is responsible for maintaining the pluripotent state of ESC in vivo. Within these genes, Oct-4, Nanog and Sox-2 form

feedforward loops, which contain two regulators that bind a set of common gene targets. This feature allows for multiple regulatory capabilities that may be responsible for ESC's ability to maintain pluripotency while also upholding the competency to properly respond to differentiation signals. In addition to their shared target genes, Oct-4, Nanog and Sox-2 regulate their own gene expression by binding to their own promoters, forming an interconnected autoregulatory loop (Boyer et al., 2005). Due to the tight framework of the feedforward and autoregulatory loop formed by Oct-4, Nanog and Sox-2, obstruction of the normal relative levels of one of these factors could alter the expression of the others, resulting in improper gene expression. For example, a normal ICM and epiblast fail to form in the absence of Oct-4 and Nanog expression. As a result, the cells from which Oct-4 was deleted no longer express Nanog, and the cells from which Nanog was deleted no longer express Oct-4 (Chambers et al., 2003). Similarly, when Sox-2 is removed from ESC, Oct-4 expression is downregulated (Masui et al., 2007) and Nanog transcription is severely compromised (Rodda et al., 2005). Nevertheless, the functional reliance upon one another is illustrative of the tight regulatory network responsible for the inherent nature of ESC. The expression of Oct-4, Nanog and Sox-2 is mandatory for the establishment and maintenance of ESC identity, and while their roles in this process are distinct, their functions are cooperative.

Expression of Oct-4, Nanog, and Sox-2 in Adult Stem Cells

The expression of Oct-4, Nanog and Sox-2 is invariably responsible for defining ESC identity; therefore, they serve as suitable markers for pluripotent cells.   Because it is known that the expression of these factors is progressively downregulated with cell differentiation, many doubted their presence in ASC. ASC are generally regarded as

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being tissue-specific, capable of generating cell lineages specific to their tissue of origin; however, recent studies have demonstrated a surprisingly high degree of plasticity among ASC. Within some ASC, researchers have detected the expression Oct-4, Nanog, and/or Sox-2, suggesting that these cells may be phenotypically and functionally similar to ESC.

Hematopoietic stem cells derived from porcine umbilical cord blood have been shown to express all three central transcription factors characteristic of pluripotent stem cells (Carlin et al., 2006). RT-PCR detected both mRNA and protein level expression of Oct-4, Nanog and Sox-2 in these cells. Furthermore, immunocytochemical analysis of Oct-4 and Nanog expression indicated that virtually all of the porcine umbilical cord cells displayed nuclear immunoreactivity for Oct-4 and Nanog (Carlin et al., 2006). While these findings suggest that these cells are phenotypically similar to pluripotent ESC, researchers lacked sufficient evidence to define the potential of these porcine umbilical cord cells. Both Oct-4 and Sox-2 have been identified in human and non-human MSC derived from bone marrow and adipose tissue (Izadpanah et al., 2006). Early passages of these MSC demonstrated that Oct-4 and Sox-2 displayed high levels of co-localization, which researchers believed may be imperative to MSC's differentiation potentials. Alternatively, these co-localization levels significantly decreased at higher passages, suggesting that gene transcription is somehow inhibited at higher passages (Izadpanah et al., 2006).

In addition to MSC from bone marrow and adipose tissue, Oct-4 expression has also been detected in the cells of the epidermis (Dyce et al., 2004), bronchial epithelium, myocardium, pancreas and testes (Ratajczak et al., 2007), disproving the

previously-held notion that Oct-4 is expressed only in the germ cells of adult animals (Schöler, 1990). Admittedly, Dyce et al (2004) were surprised by their findings, which detected the presence of Oct-4 in porcine skin-oriented sphere (PSOS) cells. From this cell population, individual PSOS cells differentiated into neuron-like, astrocyte-like, and adipocyte-like cells, inferring that a population of undifferentiated PSOC stem cells with multipotent differentiating capabilities exists within the fetal porcine skin (Dyce et al., 2004). Similar results have been reported in a population of fetal somatic stem cells, which also expressed Oct-4. In addition to various mesenchymal organs, these cells were shown to give rise to cell types of the mesodermal lineage and migrate into the genital ridge, suggesting germ-line transmission might be possible (Kues et al., 2005).

Undoubtedly, these studies have had a tremendous impact on stem cell biology. While these studies present evidence of an accessible, plentiful, and versatile source of stem cells for research and cell-based therapy, they contradict the basic principles of ASC. Obviously, this has sparked skepticism and debate among scientist, particularly concerning the inherent integrity and capability of ASC. Early reports attributed the apparent plasticity of ASC to transdifferentiation (Ferrari et al., 1998; Bjornson et al., 1999). Transdifferentiation is essentially the total conversion of a cell of one tissue lineage into a cell of a completely different lineage, with simultaneous loss of the tissue-specific markers and function of the original cell type, and attainment of the markers and function of the transdifferentiated cell type (Wagers and Weissman, 2004). This process is proposed to occur either directly or through a related process known as dedifferentiation. In this case, differentiated cells are cued to revert back to a less mature state (Raff, 2003).   Some scientists question the validity of transdifferention

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and argue that the stringent criteria for an event to be classified as such have not yet been established (Wagers and Weissman, 2004).   Instead, many propose that this observed ASC phenomena, mistakenly interpreted as transdifferentiation, is really due to spontaneous cell-cell fusion (Ying et al., 2002). Co-culturing brain cells with ESC from the mouse, Ying et al (2002) successfully isolated cells that expressed properties similar to ESC. These cells expressed Oct-4, as well as, ESC markers, alkaline phosphatase and stage-specific embryonic antigen. Furthermore, they exhibited dependency on LIF to suppress differentiation, and also demonstrated their ability to form embryoid bodies, which were capable of giving rise to neurons. Unlike ESC, however, these selected cells exhibited enlarged nuclei with multiple nucleoli, morphological features consistent with hybrid cells. Chromosome analysis of these cells revealed a tetraploid or near tetraploid compliment in these cells, leading Ying et al (2002) to conclude that these were, in fact, hybrids generated by spontaneous fusion between the mouse ESC and brain cells. Another possible explanation for the apparent plasticity of ASC is that multiple adult organs house a small population of pluripotent stem cells, which can be stimulated to proliferate and differentiate into lineages other than the tissue of origin (Jiang et al., 2002).   Jiang et al (2002) isolated these rare cells from bone marrow and demonstrated their ability to differentiate into endothelium, ectoderm and endoderm cell lineages *in vitro* and *in vivo*. While the exact mechanism responsible for this phenomenon is a source of debate among the scientific community, there is no doubt of the far-reaching abilities these ASC posses.

Induced Pluripotent Stem Cells

In 1997 Wilmut et al. introduced "Dolly", a cloned sheep produced from somatic cell nuclear transfer (SCNT), demonstrating that somatic cells can be reprogrammed when their nuclear components are transferred into oocytes. In SCNT, the nuclear components of a somatic cell are transplanted into an enucleated unfertilized ovum, which reprograms the genome of the somatic cell to an embryonic state that is capable of generating a cloned animal or producing pluripotent ESC (Wilmut et al., 1997). While much of the ensuing excitement focused on the birth of a cloned animal, many in the medical and scientific community were enthused with the prospect of producing patient-specific ESC for therapeutic use by SCNT. In recent years, however, the potential capabilities of SCNT have been overshadowed by ethical concerns as well as the inefficient nature of the procedure, leading researchers to explore alternative reprogramming methods for the generation of ESC-like cells.

The search for alternatives to SCNT underscores a great deal of the recent advancements made in stem cell research. Indeed, one of the most-publicized topics in stem cell research today is induced pluripotent stem cells (iPSC). iPSC are non-pluripotent somatic cells engineered to be pluripotent. Aside from SCNT, there are three primary mechanisms of producing iPSC: fusion with ESC, exposure to ESC extracts, and by defined factors. Needless to say, iPSC are believed to be immensely beneficial in the study and treatment of disease as the direct reprogramming of somatic cells provides an opportunity to generate patient- or disease-specific pluripotent stem cells (Nakagawa et al., 2008). While these three alternative methods of producing iPSC are designed to circumvent many of the problems associated with SCNT, they

may in fact improve the efficiency of SCNT by providing an optimal source of donor cells for reprogramming. The supposed potential of iPSC lies in the fact that these cells are morphologically similar to pluripotent ESC and, most importantly, demonstrate key characteristics of pluripotent ESC, including expressing stem cell markers, forming teratomas containing cells of all three germ layers, and contributing to multiple cell lineages (Takahashi and Yamanaka, 2006).

Wilmut et al. (1997) laid the foundation for a new area of research when they demonstrated cell fate can be reversed through SCNT. Since then, three additional methods of reprogramming the nuclei of human somatic cells have been established. The first of these reprogramming processes using human cells was introduced in 2005, when Cowan et al. revealed that fusion of human ESC with human fibroblasts cells results in hybrid cells characteristically similar to ESC. Interestingly, cell fusion experiments using cells of embryonic origin date back to the 1970's, when Miller and Ruddle showed that resulting hybrid cells formed between murine teratocarcinoma cells and thymus cells were phenotypically similar to their pluripotent embryonal carcinoma parent (Miller and Ruddle, 1976).

Over 30 years later, Rathjen et al (2002) produced similar results when they fused mouse ESC-derived neuroectoderm with undifferentiated mouse ESC, resulting in hybrid cells that expressed pluripotency-associated genes at levels comparable to those in ESC.   To test if human ESC were capable of nuclear reprogramming like their murine counterpart, Cowan et al (2005) fused human ESC with human fibroblast cells. Following fusion, hybrid cells containing both ESC and fibroblast cell chromosomes in a single nucleus were formed; however, the hybrid cells exhibited characteristics

consistent with the ESC, indicating that the phenotype of human ESC is dominant and that they are also capable of reprogramming human somatic cells (Cowan et al., 2005). Unlike the ooplasm of an enucleated oocyte in SCNT, nuclear reprogramming of a somatic cell to a less differentiated state through cell fusion is governed by ESC nuclei, which reactivates Oct-4 and silences the gene expression of the somatic cell (Do and Scholer, 2004; Do and Schöler, 2004; Alberio et al., 2006). Furthermore, DNA analysis showed that the promoter region of Oct-4 in the hybrid cells was demethylated and indistinguishable from that found in human ESC, proving that the epigenetic information controlling the transcription of pluripotency genes was reprogrammed (Cowan et al., 2005). Cell fusion for the production of iPSC is an effective and reliable alternative to SCNT; however, because the resulting hybrid cells contain an abnormal ploidy as well as nonautologous genes from the pluripotent parent, their therapeutic application is significantly hindered (Pralong et al., 2006).

Although Cowan et al. (2005) believed that their protocol for producing iPSC could be beneficial in the study and treatment of human disease, they recognized the need to eliminate the ESC chromosomes from the fusion-generated iPSC before they could be of therapeutic use. Indeed, it was experiments such as this that provided the inspiration for the development of a cell-free means to reprogramming the nuclei of somatic cells to a pluripotent state. One such way of doing this is through exposure to nuclear and cytoplasmic extracts of ESC. Ha°kelien et al (2002) had previously demonstrated the reprogramming abilities of nuclear and cytoplasmic extracts using human 293T fibroblast cells and human T cells. In addition to providing rationale for developing cell-extract based systems for reprogramming cell fate, this study developed

a highly efficient protocol for reprogramming through cell-derived extracts that has been referenced in numerous succeeding studies. Ha°kelien et al (2002) derived nuclear and cytoplasmic extracts from human peripheral blood T cells by lysing the cells from culture using a cell lysis buffer. For the 293T cells to take up the T cell extracts, their cellular membrane was permeabilized using Streptolysin O (SLO), which binds to cholesterol in the cell membrane and forms holes in the plasma membrane of the cell. The T cell extracts and the permeabilized 293T cells were incubated together for approximately 50 min and then the plasma membrane was resealed using a $CaCl_2$-containing cocktail (Ha°kelien et al., 2002). Plasma membrane resealing following SLO exposure is dependent upon Ca^{+2}, as the Ca^{+2} influx triggers a rapid repair process to the permeabilized plasma membrane (Walev et al., 2001).

A more recent study used this same method of extract derivation and membrane permeabilization to induce dedifferentiation, associated with genomewide programming of gene expression and epigenetic reprogramming of an embryonic gene, in epithelial 293T cells treated with an extract of undifferentiated human NCCIT carcinoma cells. As a result, the extracts of these ECC were able to induce markers of dedifferenation and signs of differentiation plasticity in an otherwise more developmentally restricted cell type (Taranger et al., 2005). In an effort to better understand the molecular processes underlying nuclear reprogramming, researchers built upon this study and were able to provide evidence of reprogramming of DNA methylation and histone modifications on the Nanog promoter and throughout the Oct-4 regulatory region in human epithelial cells (Freberg et al., 2007). In order to identify those cells that had been stably reprogrammed to express pluripotency-associated genes, the treated cells in all of the

aforementioned studies had to be grown in culture for several weeks, which makes it

difficult to ascertain reprogramming efficacy as well as complicating further biochemical

analysis procedures (Bru et al., 2008). In 2008, however, Bru et al. reported the

detection of key pluripotency-associated genes in ESC-extract treated cells within a few

hours of exposure, proving the first stages of reprogramming do not require a long

incubation period.   Interestingly, the expression of these genes increased in the 48

hours following exposure to extracts, indicating that long-term reprogramming of gene

expression had been induced (Bru et al., 2008). While further studies to validate

extract-based nuclear reprogramming are needed, it has proven to be an effective

means to nuclear reprogramming, which may be a more appealing option of generating

iPSC than by either SCNT or cell fusion.

One of the most significant breakthroughs in stem cell research to occur in recent

years is the production of iPSC by defined factors. Indeed, it is arguably the most

celebrated scientific advancement since the birth of "Dolly". Like the previously

mentioned methods of generating iPSC, the ectopic expression of defined transcription

factors can also generate genetically-tailored stem cells for therapeutic use by

reprogramming the nucleus of a differentiated cell to function like that of an ESC. In

contrast to the production of iPSC by SCNT, fusion with ESC or exposure to ESC

extracts, the defined factors methodology is far less controversial because it does not

make use of ESC or oocytes. Instead, this technique relies upon the actions of four

transcription factors to elicit pluripotent cells from otherwise developmentally-restricted

cells. Considering the transcription factors known to function in the maintenance of

pluripotency in ESC as well as those specifically expressed in ESC, Takahashi and

Yamanaka (2006) selected 24 genes as contenders for factors capable of inducing pluripotency in somatic cells. To determine which genes are critical for reprogramming a somatic cell to an ESC-like state, Takahashi and Yamanaka (2006) developed an assay system in which a gene's ability to induce pluripotency was determined by its ability to trigger the formation of G418-resistant colonies following its induction to mouse embryonic fibroblast cultures by retroviral transduction.   Of these 24 genes, Oct-4, Sox-2, c-Myc and Klf-4 were identified as the essential factors for generating iPSC directly from fibroblast culture (Takahashi and Yamanaka, 2006). The resultant iPSC exhibited morphological features and proliferative properties consistent with ESC, and also expressed ESC marker genes. Furthermore, subcutaneous injection of the iPSC in nude mice elicited the formation of teratomas that contained tissues from all three germ layers, indicative of the pluripotent state of the iPSC (Takahashi and Yamanaka, 2006).

The crucial roles Oct-4 and Sox-2 play in maintaining ESC identity are well established; therefore, it was not surprising to discover they also serve in the direct production iPSC in culture. However, it was surprising to find that Nanog, whose expression is essential to the pluripotent-state of ESC, was dispensable to this process, whereas c-Myc and Klf-4 were imperative (Takahashi and Yamanaka, 2006). c-Myc and Klf-4 are proposed to indirectly enhance Oct-4, Sox-2 and Nanog function, respectively, in the iPSC. It is believed that c-Myc may induce global histone acetylation in the mammalian genome (Fernandez et al., 2003), which, in turn, potentiates Oct-4 and Sox-2 target binding. Klf-4, on the other hand, may contribute to Nanog activation by repressing p53, which is known to suppress Nanog expression in ESC during differentiation (Lin et al., 2005). Indeed, the exact mechanisms these

factors employ to induce pluripotency in somatic cells is undetermined, however, their ability to do so is universal. Although human ESC differ from murine ESC in many aspects, Takahashi et al. (2007) reported the generation of iPSC from adult human fibroblast cultures using the same defined factors less than a year after their initial study. The functional uniformity of Oct-4, Sox-2, c-Myc and Klf-4 across species suggests that these four factors are fundamentals of a common pluripotency-governing system. Furthermore, the similarities in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, telomerase activity, and pluripotent differentiation ability noted between human iPSC and true human ESC (Nakagawa et al., 2008) are similar to those observations between murine iPSC and murine ESC (Takahashi and Yamanaka, 2006).

The ability to produce undifferentiated stem cells without the use of oocytes or any form ESC is perhaps the most realistic methodology of generating human ESC for government-approved treatment of disease and injury because it sidesteps the ethical considerations that have long hindered the therapeutic use of ESC. Because the production of iPSC by defined factors uses an individual's own cells, possible tissue rejection following transplantation is also avoided. Although the strategy developed by Takahashi and Yamanaka for generating iPSC has enormous therapeutic potential, it is not an infallible technique. The greatest problem with these iPSC is the possible induction of cancer following transplantation brought about by the use of c-Myc and retroviruses for delivering genetic material to the cells to be reprogrammed. Despite the infancy of iPSC research, many notable efforts have been made to reduce the incidence of tumorigenicity following iPSC transplantation, including the omission of c-Myc

(Nakagawa et al., 2008) and gene delivery without viral integration (Okita et al., 2008; Stadtfeld et al., 2008). Although each of these studies reported the generation of iPSC, the number of iPSC yielded in these studies was significantly less compared to the number of iPSC produced by the original method proposed by Takahashi and Yamanaka (2006). Nevertheless, the omission of c-Myc from the pool of pluripotency-inducing transcription factors as well as retroviruses for gene delivery have both proven to be effective at reducing the risk of cancerous tumor formation.

Although the primary hindrance in this methodology's application in cell based therapy has been tackled with steadfastness, there are still unresolved issues regarding the aforementioned techniques of generating iPSC by defined factors. The majority of the studies use readily-accessible fibroblast cells as the parental cells to be reprogrammed; however, these cells require a minimum of four weeks in order to be reprogrammed, resulting in an overall lengthy process. Furthermore, the terminally differentiated state of fibroblast cells may account for their relatively low reprogramming efficiency. While cell types such as hepatocytes and neural progenitors are a better fit for epigenetic reprogramming, they are impractical for human application as they are obtainable only through considerably invasive surgery (Sun et al., 2009). Although iPSC research is still in its infancy, the problems regarding the inefficient nature of the procedure, culture periods, and establishing an adequate, readily-accessible source of cells for reprogramming may already be solved. Researchers at Stanford University School of Medicine have recently reported a 20-fold more efficient production of iPSC using ASC derived from lipoaspirates of human adipose tissue at a 2-fold faster reprogramming time compared to the original report published by Takahashi and

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Yamanaka (2006). Additionally, these iPSC were generated under feeder-free

conditions using adult human ASC (hASC), which reduces the variability of

reprogramming commonly associated with mouse feeder cells (Sun et al., 2009). The

hASC were isolated from the lipoaspirates of adults between the ages of 40 and 65, and

then transduced with individual lentiviruses containing human Oct-4, Sox-2, Klf-4 and c-

Myc on two consecutive days.   Comparisons between the adult hASC-derived iPSC

and true ESC revealed the following similarities: ESC markers, relative levels of

pluripotency-associated gene expression, morphology, global mRNA patterns,

hypomethylation patterns within the Oct-4 and Nanog promoter regions and

differentiation capabilities in vivo and in vitro (Sun et al., 2009). Previous studies have

reported many of these same similarities between ESC and iPSC from fibroblast cells,

and, while there is no marked difference in hASC- and fibroblast cell- derived iPSC

ability to mimic ESC, only hASC exhibit alkaline phosphatase (AP) activity, a common

marker of pluripotency in ESC. Based on this evidence, Sun et al. (2009) speculate that

the increased efficiency and culture time of the adult hASC-derived iPSC is due to the

inherently less differentiated state of ASC, which is shown to be more like pluripotent

cells than terminally differentiated fibroblast cells.

In addition to their more suitable epigenomic regulatory pattern for

reprogramming, ASC possess other discernible advantages over other cell types for

reprogramming. ASC retain a degree of plasticity capable of multiple lineage

differentiation and are present in many tissues and organs; however, ASC reside as

very small populations in several of these sources, many of which are obtainable only

through highly invasive surgeries.   Adipose tissue, on the other hand, represents a

readily accessible and plentiful source of ASC in all individuals that is easily isolated through a safe and quick lipoaspiration procedure. Furthermore, these cells can be isolated the same day as the lipoaspiration procedure and reprogrammed immediately following seeding (Sun et al., 2009). Despite the neoteric state of this study, the feeder-free extraction of iPSC from adipose-derived adult hASC is immensely promising for the future of iPSC in research and therapy. Indeed, this methodology may prove to be a widely accepted means of generating iPSC for clinical application.

A Look Ahead

As a frontrunner in both scientific and medical studies for nearly 30 years, unwavering interests in stem cells continue to intrigue minds, excite imaginations, and, most importantly, fuel research. Unlike any other known cell type, stem cells, both ESC and ASC, can replicate themselves many times over while remaining unspecialized, yet they are capable of differentiating into multiple cell lineages. These characteristics not only define the inherent nature of stem cells, but also underline the enormous potentials regarding their use in research and medicine. Indeed, it is the supposed ability to cure incurable diseases, repair damaged organs and tissues, and heal the lame that has consistently fueled efforts in stem cell research. However, stem cells' possible applications are far-reaching, as their use has been implemented in new drug testing, improving biotechnological techniques and enhancing scientists' understanding of the molecular genetic factors responsible for normal and abnormal early embryonic development and cell differentiation (Hipp and Atala, 2008). Before stem cells can be consistently and successfully applied in any of these situations, an accessible source of competent cells must be established. The nature of ESC renders them as an ideal

source of cells for such applications; however, unresolved technical issues, possible

immune rejection following treatment, and moral discord impede the current use of

these cells. While scientific research may one day correct these existing technical and

medical impediments, the ethical debate over ESC is not likely to ever be resolved.

Alternatively, ASC are essentially accessible, safe, and incontrovertible and have been

established as a viable and successful therapy option for several years. Although the

multipotent state of ASC has proven to be sufficient for the treatment of some life-

threatening diseases such as leukemia, it lacks the ability to differentiate into lineages

outside of its host tissue, therefore, limiting ASC's therapeutic potential. Despite these

limitations, ASC are currently the most practical source of serviceable stem cells.

The fulfillment of the great promises proposed for stem cells ultimately hinges on

the sanctioned establishment of an accessible and plentiful source of unspecialized

cells capable of ubiquitous differentiation and long-term proliferation. Interestingly,

recent evidence suggests that such a stem cell population may be engineered to meet

these requirements. Reprogramming the genome of ASC to a less mature state is

currently the most promising and realistic approach towards realizing the therapeutic,

technological and educational potentials of stem cell application. The previously-

discussed methods of nuclear reprogramming rely upon a somatic cell's ability to adopt

the phenotype of ESC after one of the following procedures: SCNT (Wilmut et al.,

1997), fusion with ESC (Cowan et al., 2005), exposure to ESC extracts (Bru et al.,

2008), introduction of defined factors (Takahashi and Yamanaka, 2006).

CHAPTER III

EXPRESSION OF PLURIPOTENCY-ASSOCIATED GENES IN BOVINE FETAL FIBROBLASTS AND ADIPOSE-DERIVED ADULT STEM CELLS

Introduction

The extraordinary nature of ESC lends them the ability to give rise to all the cell types of a mammalian organism, an attribute known as pluripotency. The pluripotent state of ESC is dependent upon the expression of the genes Oct-4, Nanog and Sox-2, which have also been identified as the key transcriptional regulators of pluripotency (Boyer et al., 2005; Kuroda et al., 2005; Rodda et al., 2005). Because differentiation of ESC towards cell types of the three germ layers is triggered by the down-regulation of Oct-4, Nanog and Sox-2, they have long been considered to be ESC-specific; however, recent studies have reported the expression of these genes in some sources of ASC of both the mouse and pig (Kues et al., 2005; Carlin et al., 2006). A variety of somatic cell types from several different species, including epidermal cells, bone marrow, adipose tissue, bronchial epithelium, myocardium, pancreas, umbilical cord blood and testes, have been found to express one or more of these pluripotency-associated genes (Dyce et al., 2004; Izadpanah et al., 2006; Ratajczak et al., 2007), suggesting that these tissues and organs constitute a population of stem cells that share some phenotypic characteristics of ESC.

The differentiation potential of ASC has been thought to be limited to cell lineages present in the organ from which they are derived; however, several studies have challenged this notion by demonstrating that some ASC exhibit a remarkably high degree of plasticity. This feature allows ASC to cross lineage barriers and adopt the expression profiles and functional phenotypes of cells unique to other tissues (Herzog et

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al., 2003). Observations of ASC plasticity have been reported in several different somatic cell types, but the majority of these studies have been performed using hematopoietic and mesenchymal stem cells derived from bone marrow. Hematopoietic stem cells (HSC) can differentiate into every type of mature blood cell (Wagers and Weissman, 2004), whereas mesenchymal stem cells (MSC) are capable of differentiating towards the osteogenic, adipogenic, neurogenic, myogenic and chondrogenic lineages (Zuk et al., 2001; Mizuno et al., 2002).   The multipotent nature of MSC is believed to have several clinical applications for cell therapy and tissue engineering, and reports of a broader differentiation repertoire only add to its therapeutic potential. MSC have demonstrated efficacy as therapeutic vectors in animal models of lung injury (Ortiz et al., 2003; Ortiz et al., 2007), kidney disease (Kunter et al., 2006), diabetes (Lee et al., 2006), graft versus host disease (Ringden et al., 2006), myocardial infarction (Minguell and Erices, 2006), and various neurological disorders(Phinney and Isakova, 2005).

Although bone marrow-derived MSC have demonstrated immense therapeutic potential, their clinical use is hindered by a number of problems. Access to this cell population is obtainable only through a highly invasive and painful procedure, which yields very few MSC. Adipose tissue, on the other hand, represents a readily accessible and plentiful source of MSC that can easily be isolated. Approximately 400,000 liposuction procedures are performed in the United States annually, yielding anywhere from 100 ml to > 3 L of adipose tissue per procedure (Katz et al., 1999). This minimally invasive procedure yields an average of 400,000 cells per mL of lipoaspirate tissue in humans (Halvorsen et al., 2001; Zuk et al., 2001; Gimble and Guilak, 2003;

Aust et al., 2004). Alternatively, the volume of human bone marrow taken under local

anesthesia is typically limited to only 40 ml (Bacigalupo et al., 1992) and contains

approximately 2.4 x 10^4 MSC in total (Strem et al., 2005).

Heightened interest in the potential therapeutic use of ADAS cells has revealed a

number of similarities between bone marrow-derived MSC and ADAS cells. Like MSC

derived from bone marrow, ADAS cells have the potential to differentiate into bone,

cartilage, tendons, skeletal muscle, and fat when cultivated under lineage-specific

conditions (Zuk et al., 2001; Wagers and Weissman, 2004; Dicker et al., 2005; Lee et

al., 2006). Furthermore, comparative analysis of MSC obtained from bone marrow,

adipose tissue, and umbilical cord revealed no differences regarding morphology,

immune phenotype, success rate of isolating MSC, colony frequency, and differentiation

capabilities (Izadpanah et al., 2006; Kern et al., 2006). Perhaps the most significant

similarity between bone marrow-derived MSC and ADAS cells is their ability to suppress

immunoreactions upon transplantation. The value of bone marrow-derived MSC in

allogenic transplantations has been strengthened by reports of their

immunosuppressive properties that reduce the incidence and severity of graft versus

host disease, a major complication of allogenic hematopoietic cell transplantations

(Lazarus et al., 2000; Jorgensen et al., 2003).   Recent studies have found that ADAS

cells, like MSC derived from bone marrow, suppress immunoreactions (Puissant et al.,

2005a), indicating that ADAS cells may not elicit a cytotoxic T-cell response *in vivo*

(Gimble et al., 2007). While more comprehensive testing is needed, the field of

regenerative medicine stands to be profoundly impacted if transplanted allogenic ADAS

cells prove not to evoke an immune response resulting in rejection.   Considering the

frequency of liposuction, and the large amount of adipose tissue resulting from each procedure, it may be possible to manufacture ADAS cells in large volumes for clinical purposes. Once multiple use product approval takes place and ADAS cells manufactured in large volumes, quality assurance and control steps can be streamlined. The availability of off-the-shelf allogeneic ADAS cells will reduce the cost of cell-based therapies, and allow physicians to use them directly at the point of care rather than limiting their use to elective procedures (Gimble et al., 2007).

Recent studies have reported the presence of Oct-4, Nanog and Sox-2 in hematopoietic stem cells derived from porcine umbilical cord blood (Carlin et al., 2006). This, along with the expansive repertoire of differentiation potential exhibited by ASC, suggests these cells possess characteristics similar to pluripotent cells. Therefore, ASC may be better equipped for complete epigenetic reprogramming than terminally differentiated cells. Considering the abundance and accessibility of adipose tissue, it would be advantageous to utilize ADAS cells for nuclear reprogramming. In the present study, we used RT-PCR to analyze bovine adipose-derived adult stem (ADAS) cells and fetal fibroblast cells for the presence of the pluripotency-associated genes, Oct-4, Nanog and Sox-2.

Materials and Methods

Establishment of Primary Cultures

Primary cultures of bovine fetal fibroblasts (BFF) were established from two male fetuses between 70 and 80 days of age. A portion of the epidermis was excised from the abdominal region of each fetus, finely minced, and washed twice in a solution of

Dubelcco's phosphate buffered saline (DPBS) with Ca^{+2} and Mg^{+2}, supplemented with 2% penicillin/streptomycin (P/S) and 2% Fungizone (Gibco, 15290-018). The tissues were placed in 50 ml conical tubes containing a 0.5% collagenase solution (0.5% collagenase type I (Gibco, 17100-017) dissolved in Dulbecco's Modified Eagle Medium with high glucose (DMEM) and 1% P/S) and incubated in 5% CO_2 at 37°C for 3 h for complete enzymatic dissociation of the tissues. Following incubation, complete culture medium (DMEM with 15% fetal bovine serum (FBS) and 1% P/S) was added to each tube to inactivate the enzymatic activity of the collagenase and then centrifuged at 350 x g for 5 min. Cells were resuspended in 5 ml of complete culture medium and cultured in 75-cm^2 flasks under 5% CO_2 and 90% humidity at 39°C.

For primary cultures of adipose-derived adult stem cells (ADAS), approximately 5 g of subcutaneous adipose tissue was collected from the brisket of adult cattle at a local abattoir. The tissues were finely minced, washed in a solution of DPBS with Ca^{+2} and Mg^{+2}, 2% P/S and 2% Fungizone twice, and next transferred to sterile Erlenmeyer flasks. For enzymatic digestion, tissues were incubated in a 0.25% collagenase solution (DPBS with Ca^{+2} and Mg^{+2}, 1% Bovine Serum Albumin (BSA), 0.25% collagenase type I, 1% P/S, 1% Fungizone) for 3 h in a continuous shaking incubator. Afterwards, the samples were transferred to 50 ml conical tubes and centrifuged at 350 x g for 5 min. Residual adipose tissue was discarded from the tubes, and the cell suspensions were passed through a syringe double filter consisting of 80 μm and 120 μm nylon filters. Centrifugation was repeated, followed by a wash in DPBS with Ca^{+2} and Mg^{+2} supplemented with 1% BSA. Lastly, cells were resuspended in 1 ml of complete culture medium (DMEM, 10% FBS, 1% P/S) and cultured in 12.5-cm^2 flasks

under 5% CO_2 and 90% humidity at 39°.   After 48 h, the flasks were washed with 2 ml of DPBS with Ca^{+2} and Mg^{+2}, and 2 ml of fresh complete culture media was added before being placed back in the incubator.

Cell Culture Maintenance

Two BFF cell lines and three ADAS cell lines were established and maintained in culture for the duration of six passages. Cell cultures were passaged at 90% confluence by trypsinization. Trypsin (0.25% EDTA) was added to confluent cultures to disaggregate cells adherent to the flask, which were then counted using a hemocytometer. BFF cells were re-seeded at an initial concentration of 2.1 x 10^6 cells per 75-cm^2 flask. ADAS cells were re-seeded at an initial concentration of 0.7 x 10^6 per 25-cm^2 flask.

Cell Cryopreservation

At passages two, four and six, cells from all BFF and ADAS cell lines were frozen. Cells that were not re-seeded following trypsinization were resuspended in calf serum (CS) supplemented with 10% DMSO (dimethyl sulphoxide) (Sigma, No. D2650) and cooled at 1.0°C/min until reaching -80°C before storage in liquid nitrogen. Approximately 1,000,000 BFF cells and 300,000 ADAS cells were frozen in 1 ml of freezing medium per cryovial.   Cells were thawed as needed at room temperature for 30 sec, followed by submersion in a 38°C water bath.

Isolation of Total RNA

Total RNA was isolated from ADAS and BFF cell lines at passages two, four and six using TRI® Reagent RNA/DNA/Protein Isolation Reagent (Molecular Research Center, Inc., Cincinnati, OH). At 90% confluency, the media was removed and cells were lysed directly in the culture flask using 1 ml of TRI® Reagent per 10 cm^2. The homogenate was allowed to incubate at room temperature for 5 min and then transferred to a 15 ml phase lock gel tube. For every 1 ml of TRI Reagent used, 0.1 ml of BCP reagent (1-bromo-3 chloropropane) (Molecular Research Center, Inc., Cincinnati OH) was added to the tube and allowed to incubate at room temperature for 1 hr. The tube was then centrifuged at 12,000 x g for 15 min at 4˚C to separate the RNA from the other cellular components. After centrifugation, the clear aqueous phase containing the RNA was transferred to a 15 ml SuperClear® centrifuge tube (5 PRIME, 2302850), and the RNA was precipitated from the aqueous phase using 0.5 ml of isopropanol per 1 ml of TRI Reagent used in the initial homogenization. All samples were stored overnight at -20˚C for optimal recovery of RNA. The following day, the tube was centrifuged at 12,000 x g for 20 min at 4˚C in Sorvall RC 6 PLUS centrifuge using rotor F13-14x50cy. The RNA precipitate forms as a small white pellet on the side and bottom of the tube. The supernatant was removed, and the RNA pellet was washed in 2.5 ml of a 75% EtOH solution before being centrifuged at 14,000 x g for 10 min at 4˚C. The RNA pellet was then transferred to a 1.5 ml tube containing 1 ml of the 75% EtOH solution and centrifuged for a final time at 14,000 x g for 15 min at 4˚C. Afterwards, the supernatant was removed, and the RNA pellet was allowed to air dry. The RNA pellet was then dissolved in 50 µl of water treated with Diethyl pyrocaronate (DEPC) (Sigma, D5758)

and subjected to gel electrophoresis to confirm the presence of RNA before being stored at -80°C.

Reverse Transcription

Total RNA isolated from ADAS and BFF cell lines was reverse transcribed into cDNA in a total volume of 20 µl using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each 20 µl iScript RT Reaction mix consisted of 15 µl of RNA sample dissolved in DEPC-treated water, 1 µl of reverse transcriptase, and 4 µl of iScript reaction mix. The reaction was conducted at 25°C for 5 min, 42°C for 30 min, a denaturation step of 85°C for 5 min, and a final holding temperature of 4°C.

Reverse Transcription-PCR

All ADAS and BFF cell lines were assessed for the presence of Oct-4, Nanog and Sox-2 transcripts at passages two, four and six. Primer sets for Oct-4, Nanog, and the reference gene, polyadenylate polymerase (PAP), have previously been verified to amplify their respective transcripts in bovine embryos. All primers were designed from bovine gene sequences using the Beacon Designer 4.0 (PREMIER Biosoft International) (Table 3.1), and were diluted to 10 mM concentration. Reactions were carried out in a total volume of 25 µl, which consisted of 2.5 µl cDNA, 1 µl of each primer (sense and antisense), 12.5 µl of JumpStart™ REDTaq® ReadyMix™ PCR Reaction Mix (Sigma, No. P0982), and 8 µl of water. The program used to amplify all genes in each sample involved a denaturing cycle of 1 min at 95°C; 35 cycles of PCR (95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec); 4 min at 72°C; and a final holding temperature of 4°C. The PCR products were electrophoresed on a 2% agarose

Table 3.1. Primers used for qRT-PCR analysis

Gene	Accession Number	Primers	Amplicon Length
Oct-4	NM174580	Sense GGTTCTCTTTGGAAAGGTGTTC Antisense ACACTCGGACCACGTCTTTC	223
Nanog	DQ069776	Forward AATTCCCAGCAGCAAATCAC Reverse CCCTTCCCTCAAATTGACAC	215
Sox-2	NM001105463	Sense AGGACTGAGAGAAAGAAGAAGAG Antisense AAGAAAGAGGCAAACTGGAATC	164
PAP	X63436	Sense AAGCAACTCCATCAACTACTG Antisense ACGGACTGGTCTTCATAGC	169

gel and sequenced to confirm the correct products were amplified. To assure that the

primer sets not amplify genomic DNA, 1 ng of genomic DNA was used as a template for

the amplification of the target genes. No amplicons were recovered after RT-PCR of

genomic DNA (data not shown).

Validation of Real Time PCR

To ensure that the primers for PAP, Oct-4, Nanog and Sox-2 amplified a single

product in a quantitative manner, amplification efficiency and a correlation coefficient

from a standard curve was determined for each gene using qRT-PCR (Figure 3.1).

Purelink™ PCR Purification Kit (Invitrogen, K3100-01) was used to purify PCR products

in order to individually optimize each transcript in the amplified calibrator cDNA. A

standard curve for each gene was generated from the purified PCR product at six

different 10-fold dilutions. The calibrator cDNA was produced from a mixture of RNA

from two BFF cell lines at four different passages that was reverse transcribed into

cDNA using the same method previously described. All of the target genes had

acceptable efficiencies (80 to 120%) and correlation coefficients (close to 1.0).

Quantitative Real Time PCR

The expression levels of Oct-4, Nanog and Sox-2 in BFF cells treated with ADAS

cell extracts were quantified using The LightCycler® 480 Real-Time PCR System

(Roche Applied Science, Indianapolis, IN, USA). cDNA was amplified using SsoFast™

EvaGreen supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The total 20 µl

real time PCR mix consisted of 2 µl of cDNA, 10 µl of SsoFast™ EvaGreen Supermix,

6 µl of nuclease-free water, and 1 µl of forward and reverse primer pairs (10 mM) for
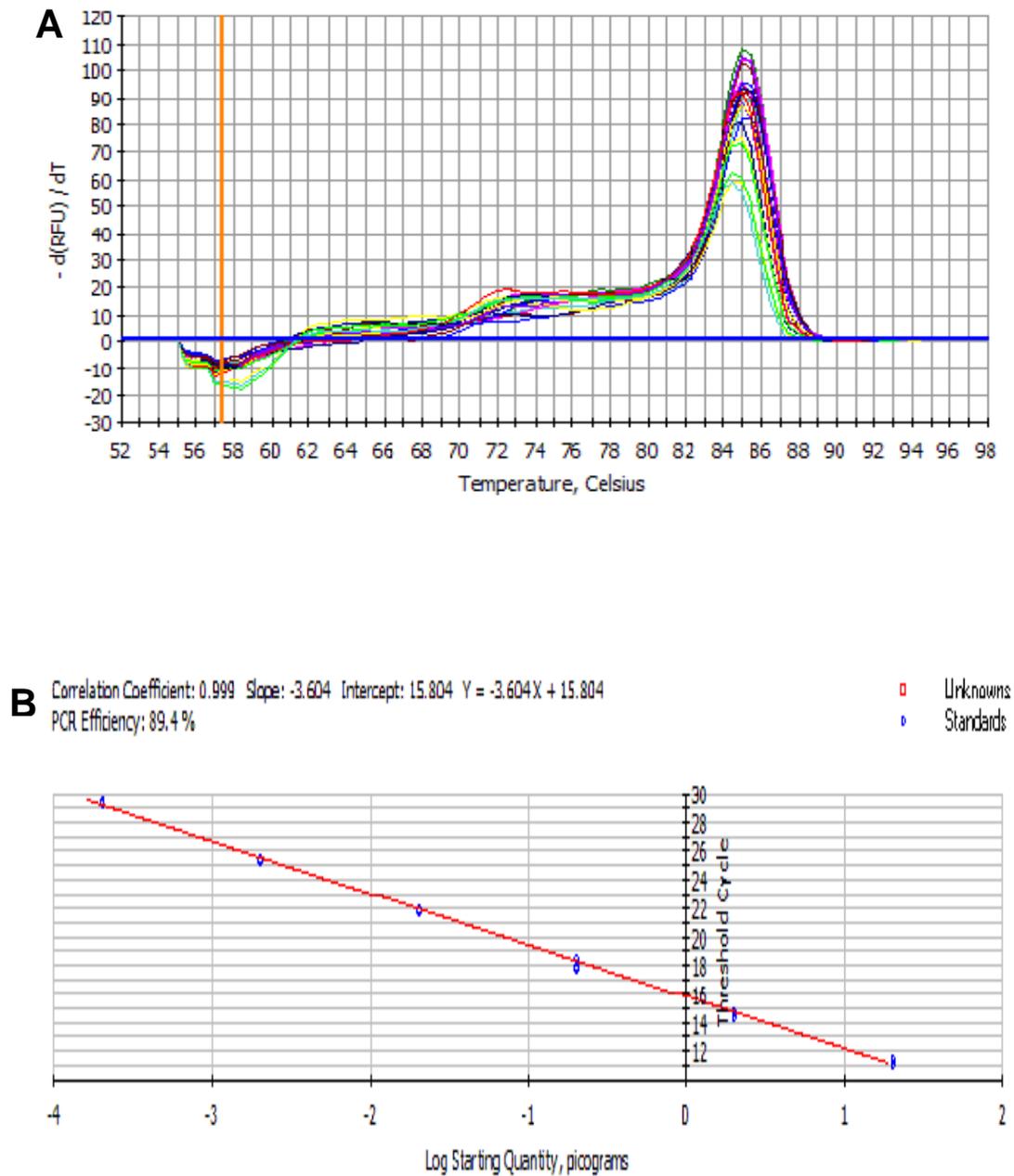
Figure 3.1. Melting curves and standard curve obtained using primers (sense and antisense) for the amplification of Sox-2. Six 10-fold dilutions of purified PCR product from calibrator cDNA were used to generate melting curves **(A)** and a standard curve **(B)**.

each gene. Within each qRT-PCR plate setup, reactions for the reference gene and each gene of interested were performed using the calibrator cDNA, the sample cDNA, and a no template negative control. The PCR program used for the amplification of all genes consisted of a denaturing cycle of 30 sec at 95°C; 45 cycles of PCR (95°C for 5 sec and 55°C for 20 sec); a melting curve analysis which consisted of 95°C for 5 sec, 65°C for 1 min, followed by continuous acquisition at 97°C, with 5 acquisitions per °C; and a final holding temperature of 40°C.

Data was quantified using the method for relative quantification in qRT-PCR described by Pfaffl (2001). Values are reported as relative transcription or the n-fold difference relative to a calibrator. A mixture of cDNA from BFF cells at multiple passages was used as a calibrator for all of the target genes. PAP was used as the internal reference gene. The threshold cycle (Puissant et al.) value of the reference gene was used to normalize the target gene signals in each sample. The amount of target transcripts relative to the calibrator was calculated using the following equation: n-fold difference = Efficiency Target Gene$^{\Delta C_T T}$/ Efficiency Reference Gene$^{\Delta C_T R}$, where an efficiency of 2 was assumed. The $\Delta C_T T$ (for the target gene) value was calculated by subtracting the sample C_T value of the target gene from the calibrator C_T value of the target gene. The $\Delta C_T R$ (for the reference gene) value was calculated by subtracting the sample C_T value of the reference gene PAP from the calibrator C_T value of the reference gene. Therefore, all target abundance levels were expressed as n-fold differences relative to a calibrator and normalized to the reference gene in order to compensate for PCR variations between runs.

Results

The presence of Oct-4, Nanog and Sox-2 transcripts in all BFF and ADAS samples at passages two, four and six was confirmed by gel electrophoresis following RT-PCR (Figure 3.2). qRT-PCR was subsequently employed in order to quantify Oct-4, Nanog and Sox-2 expression levels in these samples. The relative expression level of each gene was determined by calculating the [delta][delta]C_t of each gene relative to the calibrator for the given gene and normalized to the reference gene, Poly A Polymerase (Tables 3.2 and 3.3). The relative expression levels of Oct-4, Nanog and Sox-2 in BFF and ADAS cell samples were generally low, but marked variations in these expression levels were observed between cell lines and passage numbers. Notably, Oct-4, Nanog and Sox-2 expression in ADAS#1 at passage two was consistently higher than that of the calibrator at passage two; however, gene expression was noticeably reduced in passages four and six.

Discussion

The differentiation potential of adult stem cells (ASC) has long been thought to be limited to cell lineages present in the organ from which they are derived; however, several studies have challenged this notion by demonstrating that some ASC exhibit a particularly high degree of plasticity. Unlike terminally differentiated somatic cells, the less differentiated state of ASC can assume the functional phenotypes and expression profiles of cells unique to other tissues (Herzog et al., 2003). The expansive repertoire of differentiation potential exhibited by ASC suggests these cells possess characteristics similar to pluripotent cells. Recent studies reporting the presence of Oct-4, Nanog and Sox-2 expression in some sources of ASC of both the mouse and pig

A

B

C

D
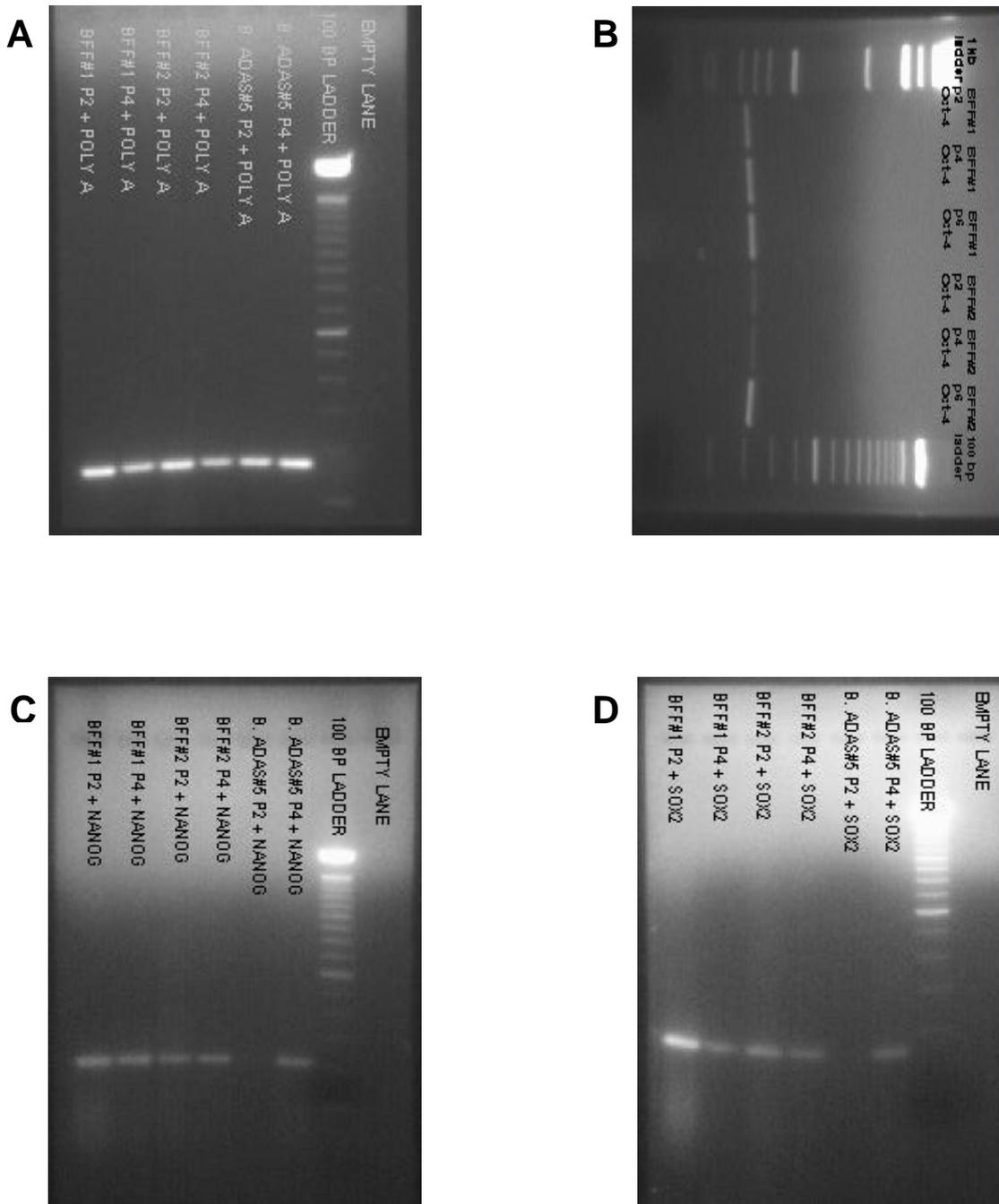
Figure 3.2. Gel Electrophoresis images confirming the presence of
Poly A **(A)**, Oct-4 **(B)**, Nanog **(C)** and Sox-2 **(D)** in ADAS and BFF cells.

Table 3.2. Oct-4, Nanog and Sox-2 relative expression levels in ADAS#1, ADAS#2 and ADAS#3 at passages two, four and six[1]

Sample	Oct-4	Nanog	Sox-2
ADAS#1 p2	79.88	138.80	76.58
ADAS#1 p4	$1.28E^{-4}$	$1.53E^{-4}$	$1.60E^{-4}$
ADAS#1 p6	$2.15E^{-3}$	$1.36E^{-3}$	$4.12E^{-4}$
ADAS#2 p2	$2.16E^{-7}$	14.86	15.36
ADAS#2 p4	$7.57E^{-3}$	$1.44E^{-4}$	$3.60E^{-3}$
ADAS#2 p6	$3.53E^{-3}$	$1.47E^{-3}$	$4.49E^{-3}$
ADAS#3 p2	$1.87E^{-4}$	$9.73E^{-5}$	$4.30E^{-6}$
ADAS#3 p4	$5.41E^{-4}$	$7.82E^{-5}$	$8.38E^{-5}$
ADAS#3 p6	$5.51E^{-2}$	$1.70E\text{-}^{3}$	$5.73E^{-4}$

[1]The three ADAS cell lines analyzed are designated as ADAS#1, ADAS#2 and ADAS#3. Relative expression of Oct-4, Nanog and Sox-2 was measured in all three of these samples at passages two, four and six, denoted as p2, p4 and p6, respectively.

Table 3.3. Oct-4, Nanog and Sox-2 relative expression levels in BFF#1 and BFF#2 at passages two, four and six[1]

Sample	Oct-4	Nanog	Sox-2
BFF#1 p2	0.13	$2.69E^{-5}$	0.08
BFF#1 p4	0.03	0.19	0.05
BFF#1 p6	2.12	23.93	$2.69E^{-5}$
BFF#2 p2	0.03	0.19	0.06
BFF#2 p4	$3.32E^{-4}$	$3.88E^{-4}$	$1.04E^{-4}$
BFF#2 p6	$6.58E^{-4}$	0.85	$1.10E^{-3}$

[1]The two BFF cell lines analyzed are designated as BFF#1 and BFF#2. Relative expression of Oct-4, Nanog and Sox-2 was measured in both of these samples at passages two, four and six, denoted as p2, p4 and p6, respectively

(Kues et al., 2005; Carlin et al., 2006) provide further evidence in support of phenotypic and functional similarities between ASC and ESC. It should be noted that, although numerous studies have reported the expression of one or more pluripotency-associated genes in several sources of ASC, the biological and functional significance of their expression in these cells remains largely unknown. In the present study, we analyzed bovine ADAS cell and BFF cells for the presence of the pluripotency-associated genes, Oct-4, Nanog and Sox-2.

Like other studies that have analyzed sources of ASC for Oct-4, Nanog and Sox-2 expression, we report the presence of Oct-4, Nanog and Sox-2 transcripts in bovine ADAS cells and BFF cells (Dyce et al., 2004; Kues et al., 2005; Carlin et al., 2006; Izadpanah et al., 2006; Ratajczak et al., 2007). While transcripts for all three pluripotency-associated genes were detected in all samples, we found their expression to be highly variable between cell lines and passage numbers.   It should be noted that among these studies, only the study by Carlin et al. (2006) reported the presence of all three genes. The other studies only reported detecting one or two of these genes; however, not all these studies analyzed cells for Oct-4, Nanog and Sox-2 expression. In these studies, ASC were evaluated and found positive for the expression of other pluripotency-associated genes, such as Stat3 (Dyce et al., 2004; Kues et al., 2005), Rex-1 (Izadpanah et al., 2006), and Akp2 (Kues et al., 2005). In addition, many of these studies extended their analysis of ASC beyond gene expression and presented evidence of multilineage differentiation (Dyce et al., 2004; Kues et al., 2005; Izadpanah et al., 2006), extended self-renewal capabilities (Dyce et al., 2004; Kues et al., 2005), alkaline phosphatase activity (Carlin et al., 2006), clonal-generating capacity (Dyce et

al., 2004), and telomerase activity (Izadpanah et al., 2006). While these types of tests are beyond the scope of our study, it is plausible to speculate that bovine ADAS cells BFF cells also demonstrate these characteristics.

The presence of Oct-4, Nanog and Sox-2 transcripts in cells which are not inherently pluripotent leads us to believe that there may be another level of regulation at the translational level for these genes. Although our study did not examine BFF cells and ADAS cells for the expression of these proteins, a study by Page et al. (2009) provides evidence in support of our belief. Page et al. (2009) reported basal expression of mRNA for Oct-4, Nanog and Sox-2 in primary adult human fibroblasts; however, neither Western blot analysis nor Immunocytochemistry detected the presence of these proteins. Interestingly, Oct-4, Nanog and Sox-2 proteins were detected in adult human fibroblasts cultured in a reduced oxygen atmosphere and in the presence of FGF2. FGF2 has been found to be essential in the maintenance of Oct-4, Nanog and Sox-2 expression and pluripotency (Levenstein et al., 2006). While Page et al. (2009) had not yet determined the functional relationship between FGF2 and stem cell expression in dermal fibroblasts, they did demonstrate that modifications to the *in vitro* culture environment triggered translation of Oct-4, Nanog and Sox-2 and their appropriate translocation to the cells' nuclei. Importantly, their results suggest that alteration of cell fate may depend not only on the induction of new transcription, but also on the mechanisms regulating posttranscriptional modifications and translation (Page et al., 2009).

The results of the present study support the notion that BFF cells and ADAS cells are less differentiated than other somatic cells. It has been suggested that the genome

of less differentiated cells may be more amenable to reprogramming or require less reprogramming following the induction of pluripotency either by SCNT or another experimental strategy (Rideout et al., 2001). Currently, the overall efficiency of SCNT is between 0-3% (number of live offspring as a percentage of the number of nuclear transfer embryos) (Paterson et al., 2003). While several factors have been identified as contributors to the inefficiency of the procedure, incomplete epigenetic reprogramming is considered the primary reason for developmental failure of SCNT embryos (Li et al., 2003). Because of the high degree of plasticity demonstrated by ADAS cells, it is likely that these cells have an epigenomic regulatory pattern that is closer to pluripotent ESC than terminally differentiated somatic cells (Sun et al., 2009). Therefore, the unique epigenetic landscape of ADAS cells may present fewer barriers for reprogramming, resulting in an increase in the efficiency of reprogramming techniques.

CHAPTER IV

CELL EXTRACT-BASED NUCLEAR REPROGRAMMING OF SOMATIC CELLS

Introduction

In its' simplest form, the term nuclear reprogramming has been defined as the molecular dominance of one distinct cell type over another (Western and Surani, 2002). Because the goal of most reprogramming studies is to reinstate developmental pluripotency in differentiated somatic cells, the term has come to specifically describe the reversal of the differentiated state of a mature cell to one that is characteristic of the undifferentiated embryonic state (Hochedlinger and Jaenisch, 2006). To date, the majority of reprogramming studies employ the use of SCNT; however, the relatively inefficient nature of SCNT has overshadowed its benefits and limited its application. While several factors have been identified as contributors to the inefficiency of the procedure, incomplete epigenetic reprogramming is considered the primary reason for developmental failure of SCNT embryos. The need for a more amendable and efficient means of reprogramming resulted in the development of three additional experimental approaches. Aside from SCNT, there are three experimental strategies used to induce pluripotency in differentiated somatic cells: fusion with ESC (Cowan et al., 2005), exposure to ESC extracts (Bru et al., 2008), and by defined factors (Takahashi and Yamanaka, 2006). Takahashi and Yamanaka (2006) not only provided a fourth approach to successful nuclear reprogramming, they also coined the term "induced pluripotency stem cell" (iPSC). iPSC specifically refers to the generation of pluripotent stem cells from non-pluripotent cells by induced gene expression; however, the term is

now commonly used to refer to all non-pluripotent cells that have been engineered to become pluripotent, regardless of which reprogramming method is employed.

In all of the aforementioned reprogramming strategies, differentiated somatic cells are reverted to an ESC-like state as a result of changes in the epigenome. Epigenetic changes are heritable modifications to DNA or chromatin that allow differentiated cells to perpetuate the molecular memory needed to retain their identity (Tada et al., 1997; Jones and Takai, 2001). Although epigenetic modifications are heritable, all four reprogramming methodologies have demonstrated that these modifications can be experimentally reversed. Histone modifications and DNA methylation are two of the major epigenetic modifications that play a significant role in regulating gene expression. The principle function of epigenetic modifications is the regulation of repressed genes not required in specific cell types at specific stages in development (Wolffe and Matzke, 1999). For example, when the cells of the ICM differentiate towards their specific lineages, the promoter regions of the transcription factors Oct-4, Nanog and Sox-2 are methylated, rendering them transcriptionally inactive (Nichols et al., 1998; Chambers et al., 2003; Okumura-Nakanishi et al., 2005; Yeo et al., 2007). In order to reinstate Oct-4, Nanog and Sox-2 expression, the opposite epigenetic modification, demethylation, must occur. Studies have shown partial DNA demethylation in restricted areas of the Oct-4 regulatory region as well as demethylation of the Nanog promoter in iPSC produced by SCNT, fusion with ESC, and by defined factors (Tada et al., 1997; Cowan et al., 2005; Takahashi and Yamanaka, 2006; Blelloch et al., 2007). Similarly, Freberg et al., (2007) reported demethylation throughout the Oct-4 regulatory region and the Nanog promoter in 293T cells treated

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with ESC extracts, which, along with histone modifications, resulted in the up-regulation of Oct-4 and Nanog. Although functional reprogramming of somatic cell nuclei towards pluripotency involves several complex molecular events and epigenetic changes, it is evident that demethylation is a primary epigenetic determinant in nuclear reprogramming, irrespective of the approach. Therefore, each reprogramming strategy must possess the necessary regulatory components required to elicit demethylation as well as the other appropriate epigenetic changes responsible for reinstating pluripotency in differentiated somatic cells.

Nuclear reprogramming techniques are believed to be immensely beneficial in the study and treatment of disease as the direct reprogramming of somatic cells provides an opportunity to generate patient- or disease-specific pluripotent stem cells (Nakagawa et al., 2008). While all four experimental techniques have demonstrated the ability to successfully reprogram gene expression and promote pluripotency in terminally differentiated cells (Thomson et al., 1998; Cowan et al., 2005; Takahashi and Yamanaka, 2006; Bru et al., 2008), not all of the methods are suitable for generating iPSC for clinical use. Aside from the obvious ethical issues regarding nuclear reprogramming by SCNT and fusion with ESC, major technical obstacles impede their clinical applications. The inefficient nature of SCNT, coupled with the fact that it is a technically challenging procedure, make it unlikely that SCNT could be performed on a large scale to derive pluripotent cell lines routinely for every patient (Amabile and Meissner, 2009). Although nuclear reprogramming by fusion with ESC circumvents these hindrances, abnormal ploidy and the presence of nonautologous genes from the pluripotent parent cells prevent the therapeutic use of pluripotent hybrid cells (Pralong et

al., 2006). For this approach to be viable, a practical means of removing the nucleus of

the ESC from the hybrid cell must be developed in order to generate diploid customized

cells for transplantation therapy. It will be difficult, if not impossible, to selectively

eliminate the entire set of ESC chromosomes from the hybrid cells if future research

determines DNA replication is required for reprogramming (Hochedlinger and Jaenisch,

2006).

Progress in the science of SCNT and ESC cell fusion-based reprogramming may

make it possible to overcome these technical obstacles; however, the therapeutic

potential of these reprogramming techniques will most likely continue to be hindered by

controversy. Nonetheless, the value of these techniques should not be overlooked.

Indeed, it is years of SCNT experiments that have proven the nucleus of most, if not all,

adult cells retains nuclear plasticity and can be reset to an embryonic state (Amabile

and Meissner, 2009). This, along with logistical and legislative limitations, fueled the

development of alternative reprogramming mechanisms. While these techniques were

originally designed to circumvent the problems associated with SCNT, they may in fact

improve the efficiency of SCNT by providing an optimal source of donor cells for

reprogramming. Currently, the overall efficiency of SCNT is between 0-3% (number of

live offspring as a percentage of the number of nuclear transfer embryos) (Paterson et

al., 2003). However, blastocyst rates and the number of live births have been shown to

dramatically increase when ESC are used as donor cells (Rideout et al., 2001),

suggesting that the genome of less differentiated cells is better equipped for complete

epigenetic reprogramming than that of a differentiated somatic cell. Although SCNT is

not suitable for generating therapeutic cells, the ability to produce genetically superior

livestock has established SCNT as an invaluable tool in commercial livestock production. If cell extracts or defined transcription factors are used to reprogram the somatic cells to be used as donor cells to a less differentiated state, the efficiency of cellular reprogramming during SCNT may be enhanced.

Extract-based nuclear reprogramming of differentiated somatic cells is an attractive approach towards the production of iPSC for several reasons. Aside from providing optimal donor cells for SCNT, *in vitro* reprogramming using cell extracts presents innovative technological and commercial benefits. Two advantages of extract-mediated nuclear reprogramming are the absence of introduction of ESC chromosomes into the cell to be reprogrammed, and the possibility of identifying reprogramming factors and mechanisms of reprogramming (Collas, 2007). Extract-derived factors are presumably not permanently active in target cells but turn over at kinetics corresponding to their half-lives. By circumventing the use of whole cells, the difficulties associated with removal of extra chromosomes are eliminated (Dittmar et al., 2009). Furthermore, the use of permeabilized cells allows the reprogramming factors to access the interior directly, which may not only be more effective but has the advantage of being useful without having a great deal of prior knowledge of regulatory mechanisms controlling cell function. From a commercial standpoint, extract-based reprogramming is far more practical than SCNT. Cells are the source of reprogramming material, which, unlike oocytes, can be grown in large numbers, and, if necessary, can be transformed to produce a consistent supply of reprogramming material. Importantly, *in vitro* reprogramming may be applied to many cell types and thus has potential to treat many diseases (Collas, 2007).

The hunt for a pluripotent stem cell suitable for therapeutic applications underscores the bulk of nuclear reprogramming studies. Reprogramming somatic cells towards pluripotency by exposure to ESC extracts avoids ethical and legal issues regarding human cloning and the production of ESC from human embryos because it does not use oocytes or intact ESC (Collas, 2007); however, the use of extracts from ESC is still somewhat controversial. Nevertheless, it may still be possible to produce replacement cells suitable for therapeutic applications by extract-based reprogramming. Because the genome of ASC is inherently less differentiated than other somatic cells, exposing them to extracts of other ASC may drive them further towards an undifferentiated state. Although this treatment will not produce cells that mimic true ESC, it has been suggested that it may not be necessary to produce a fully reprogrammed cell for cell replacement therapeutic applications (Collas, 2007). Extensive studies are required to validate this hypothesis, and a great deal more work is needed before this or any reprogramming technique is systematically employed to produce cells for therapeutic applications.

Extract-based reprogramming using ASC is a promising and plausible approach towards the production of replacement cells for therapeutic purposes; however, it may be several years before this reprogramming technology is applied in a clinical setting. The field of animal agriculture, on the other hand, may be able to reap the rewards of this reprogramming technique soon. In addition to enhancing commercial livestock production, SCNT is an ideal procedure for introducing specific genetic modifications in farm animals. The production of transgenic animals not only provides a means of studying genes involved in a variety of biological systems and disease processes, but

transgenic animals may also be used as bioreactors for the production of pharmaceuticals and perhaps even serve as organ donors for the human population (Edwards et al., 2003). Moreover, SCNT has been used to successfully generate histocompatible tissues, addressing one of the major challenges in transplantation medicine (Lanza et al., 2002). The benefits of SCNT, however, are often overshadowed by the relatively inefficient nature of the procedure. Incomplete epigenetic reprogramming is considered the major cause of the developmental failure of cloned embryos (Li et al., 2003). This is likely due to the extensive chromatin modifications characteristic of terminally differentiated somatic cells. It has been suggested that the nucleus of a less differentiated cell may be more amenable to or require less reprogramming than the nucleus of a fully differentiated somatic cell (Rideout et al., 2001). Because ASC are inherently less differentiated than other somatic cells, readily reverting their genome to an even less differentiated state may be possible through extract exposure.

Considering the abundance, accessibility, and differentiation capabilities demonstrated by cells isolated from adipose tissue, we believe adipose-derived adult stem (ADAS) cells are an ideal candidate for extract-based reprogramming. This belief is further supported by our findings which confirm the presence of Oct-4, Nanog, and Sox-2 in bovine ADAS cells. Currently, the vectors available for these transcription factors are either mouse or human, and the response between species is not known. Based on this, we hypothesize that nuclear and cytoplasmic extracts derived from ADAS cells possess the ability to increase Oct-4, Nanog and Sox-2 expression levels in other ASC.

Materials and Methods

Experiment 1 Experimental Design

Nuclear and cytoplasmic extracts were derived from four different ADAS cell lines at passage two. These ADAS cell extracts, which served as the reprogramming material in our extract-based reprogramming system, were incubated with reversibly-permeabilzed cells of two different BFF cell lines at various passages. Following incubation, the membranes of the permeabilized BFF cells were resealed, and the cells were allowed to culture for 4-5 days in complete culture medium. Oct-4, Nanog and Sox-2 expression in these cells was determined using qRT-PCR.

Experiment 2 Experimental Design

To further investigate the extract-based reprogramming system, BFF cells were also exposed to nuclear and cytoplasmic extracts derived from hESC. Extracts were derived from a single hESC line, which had been expanded and cultured under standard ESC culture guidelines. Cells from the same BFF cell lines were reversibly permeabilized before incubating in hESC extracts. After resealing the plasma membrane of the BFF cells, the cells were plated and allowed to culture for approximately 5 days. Oct-4, Nanog and Sox-2 expression levels in these cells were determined using qRT-PCR.

Establishment of Primary Cultures

Primary cultures of bovine fetal fibroblasts (BFF) were established from two male fetuses between 70 and 80 days of age. A portion of the epidermis was excised from

the abdominal region of each fetus, finely minced, and washed twice in a solution of Dulbecco's phosphate-buffered saline (DPBS) with Ca^{+2} and Mg^{+2}, supplemented with 2% penicillin/streptomycin (P/S) and 2% Fungizone (Gibco, 15290-018). The tissues were placed in 50 ml conical tubes containing a 0.5% collagenase solution (0.5% collagenase type I (Gibco, 17100-017) dissolved in Dulbecco's Modified Eagle Medium with high glucose (DMEM) and 1% P/S) and incubated in 5% CO_2 at 37°C for 3 h for complete enzymatic dissociation of the tissues. Following incubation, complete culture medium (DMEM with 15% fetal bovine serum (FBS) and 1% P/S) was added to each tube to inactivate the enzymatic activity of the collagenase and then centrifuged at 350 x g for 5 min. Cells were resuspended in 5 ml of complete culture medium and cultured in 75-cm^2 flasks under 5% CO_2 and 90% humidity at 39°C.

For primary cultures of adipose-derived adult stem cells (ADAS), approximately 5 g of subcutaneous adipose tissue was collected from the brisket of adult cattle at a local abattoir. The tissues were finely minced, washed in a solution of DPBS with Ca^{+2} and Mg^{+2}, 2% P/S and 2% Fungizone twice, and transferred to sterile Erlenmeyer flasks. For enzymatic digestion, tissues were incubated in a 0.25% collagenase solution (DPBS with Ca^{+2} and Mg^{+2}, 1% bovine serum albumin (BSA), 0.25% collagenase Type I, 1% P/S, 1% Fungizone) for 3 h in a continuous shake incubator. Afterwards, the samples were transferred to 50 ml conical tubes and centrifuged at 350 x g for 5 min. Residual adipose tissue was discarded from the tubes, and the cell suspensions were passed through a Millipore double filter consisting of 80 µm and 120 µm nylon filters. Centrifugation was repeated, followed by a wash in DPBS with Ca^{+2} and Mg^{+2} supplemented with 1% BSA. Lastly, cells were resuspended in 1 ml of

complete culture medium (DMEM, 10% FBS, 1% P/S) and cultured in 12.5-cm^2 flasks

under 5% CO_2 and 90% humidity at 39° C.   After 48 h, the flasks were washed with 2

ml of DPBS with Ca^{+2} and Mg^{+2}, and 2 ml of fresh complete culture media was added

before being placed back in the incubator.

Cell Culture Maintenance

BFF and ADAS cell cultures were passaged at 90% confluence and maintained

for the duration of six passages. Trypsin (0.25% EDTA) was added to confluent

cultures to disaggregate cells adherent to the flask, which were then counted using a

hemocytometer. BFF cells were re-seeded at an initial concentration of 2.1 x 10^6 cells

per 75-cm^2 flask. ADAS cells were re-seeded at an initial concentration of 0.7 x 10^6 per

25-cm^2 flask.

hESC were provided by Dr. Kenneth Eilertsen's laboratory at Pennington

Biomedical Research Center. Frozen hESC were quickly thawed in a 37°C water bath,

and immediately transferred to a sterile 50 ml tube. A total volume of 10 ml of

mTeSR$^®$1 (STEMCELL Technologies Inc., Vancouver, BC, Canada) complete culture

medium was added drop wise to the tube, and then centrifuged at 200 x g for 4 min.

The supernatant was discarded, and the cell pellet was resuspended in 2 ml of

mTeSR$^®$1. The cell suspension was transferred to 1 well of a 6-well plate that

contained prepared mouse embryonic fibroblast (MEF) feeder layer cells and placed in

a 37°C incubator. The media was replaced with fresh mTeSR$^®$1 media daily.

hESC colony growth was monitored daily to ensure the cells remained

undifferentiated. Before the colonies contacted each other, they were passaged using

dispase (1 mg/ml). Approximately 1ml of dispase was added to each well of a 6-well

plate, and then placed in a 37˚C incubator for 7 min. Afterwards, the dispase solution

was aspirated, and the wells were washed twice in 2 ml of DMEM/F12. A volume of 2

ml of mTeSR was added to each well, and the hESC colonies were scrapped from the

plate using a cell scrapper. The detached cell aggregates were transferred to a sterile

15 ml tube. Each well was rinsed with an additional 2 ml of mTeSR and added to the 15

ml tube. The hESC were evenly split into each well of a 6-well plate coated with

Matrigel and placed back in the incubator. Because residual MEF cells were likely

present in this initial hESC population, the cells were passaged twice before extracts

were derived.

Matrigel-coated plates were prepared by mixing thawed BD Matrigel™ Basement

Membrane Matrix (BD Biosciences, Franklin, NJ, USA) with approximately 25 ml of

DMEM/F12. A total volume of 1 ml of the diluted Matrigel solution was added to each

well of a 6-well plate and allowed to incubate at RT for at least 1 hr before plating hESC.

Derivation of Cell Extracts

Nuclear and cytoplasmic extracts were derived from ADAS cell lines at passage

two. At 90% confluency, cells were trypsinized and centrifuged at 300 x g for 10 min at

4˚C. Alternatively, hESC were harvested using dispase (1 mg/ml) and centrifuged at

300 x g for 5 min at RT. All cells were washed twice in ice-cold DPBS with Ca^{+2} and

Mg^{+2}, and then resuspended in approximately 9.5 ml of ice-cold Cell Lysis Buffer

without Halt™ Protease Inhibitor Cocktail (100 mM HEPES, pH 8.2, 50 mM NaCl, 1 mM

dithioreitol (DTT), 0.1 phenylmethylsulfonyl fluoride (PMSF), 5 mM $MgCl_2$, Milli-Q H_20).

The cell suspension was centrifuged at 300 x g for 10 min at 4˚C, and the resulting supernatant was discarded. To initiate cell lysis, cells were resuspended in 250 µl of cell lysis buffer containing 10 µl of Halt™ Protease Inhibitor Cocktail (100 X) (Thermo Scientific, 1860932) and 5 µl of 0.5 M EDTA and held on ice for approximately 45 min. The cell suspension was then transferred to a glass pestle on ice for Dounce Homogenization. When at least 90% of the cells were lysed, the cell lysate was transferred to pre-chilled 1.5 ml tubes and centrifuged at 15,000 x g for 15 min at 4˚C. The supernatant (extract) was collected, and 100µl aliquots were prepared and held on ice.

Extract Toxicity Assessment

A cell toxicity test was performed for each extract preparation. To test the extracts, approximately 50,000 adult bovine fibroblast cells were placed in a 1.5 ml tube with 30 µl of extract and incubated for 1 h in a 37˚C water bath. A 3 µl sample was then placed on a microscope slide and cell morphology was assessed (Figure 4.1).

Cell Permeabilization Assay

Because successful reversible permeabilization of cells with Streptolysin O (SLO) requires selection of the correct toxin, we first sought to identify the SLO concentration that results in permeabilization of 60 to 80% of the total cell population within 10-15 min (Walev et al., 2001). A 10 µg/ml stock solution of SLO was prepared by dissolving the SLO in 10 mM of DTT, and 10 µL aliquots were prepared. The SLO stock solution was then diluted in DPBS without Ca^{+2} and Mg^{+2} at a ratio of 1:5, 1:10, 1:20, and 1:30. The
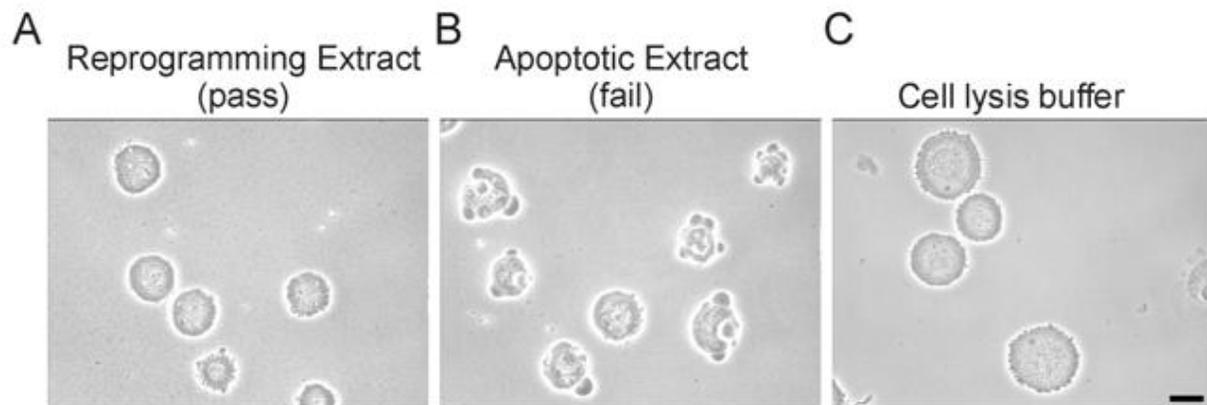
Figure 4.1. Intact fibroblast cells were placed in a 1.5 ml tube with 30 μl of extract and incubated for 1 hr in a 37˚C water bath. A 3 μl sample was then placed on a microscope slide and cell morphology was assessed. Cells shown in (A) survived exposure to the extract whereas cells in (B) did not survive extract exposure and will die in subsequent culture. Batches of extract giving rise to such cells should be discarded. Cells shown in (C) were exposed to cell lysis buffer alone.

(www.collaslab.com)

remaining SLO stock solution aliquots were stored at -20˚C. Adult bovine fibroblast

cells were harvested as per standard procedure, and aliquots of approximately 170,000

adult bovine fibroblast cells were resuspended in 488 µl of ice-cold DPBS without Ca^{+2}

and Mg^{+2} containing 50 µg/ml of propidium iodide. The cell suspensions were placed in

a 37˚C water bath for 2 min, and then individually treated with 12 µl of either the 1:5,

1:10, 1:20, or 1:30 SLO dilutions.   A fifth aliquot of 170,000 adult bovine fibroblast cells

served as a control, and, therefore, did not receive either SLO or DPBS without Ca^{+2}

and Mg^{+2}. After incubating for 50 min in a 37˚C water bath, the cells were centrifuged at

300 x g for 5 min at 4˚C, and the supernatant was discarded. To initiate resealing of the

plasma membrane, the cells were resuspended in 1.5 ml of complete culture medium

containing 2 mM $CaCl_2$ (DMEM with 10% FBS and 1% P/S). Each 1.5 ml cell

suspension was evenly distributed between 3 wells of a 24-well plate and allowed to

culture for 1 h in a 5% CO_2 incubator. Cells were observed by epifluorescence

microscopy for the uptake of propidium iodide, which is indicative of successful

permeabilization, resealing, and reseeding. SLO concentrations that resulted in the

positive staining of 60 to 80% of the cells were selected. Based on the results of the

assay, we concluded that SLO stock diluted 1:20 in DPBS without Ca^{+2} and Mg^{+2} was

sufficient for our experimentations.

Reversible Permeabilization of Donor Cells

Prior to harvesting BFF cells for reversible permeabilization, a 10 µl aliquot of the

SLO stock was removed from the -20˚C freezer and allowed to thaw at room

temperature. The SLO working solution used for cell membrane permeabilization was

prepared by diluting the SLO stock 1:20 in ice-cold DPBS without Ca^{+2} and Mg^{+2} and held on ice until use.

BFF cells used as donor cells were harvested by the same methods previously described. The cells were washed in 10 ml of ice-cold DPBS without Ca^{+2} and Mg^{+2} and centrifuged at 300 x g for 10 min at 4°C. Because Ca^{+2} inhibits SLO activity, the cells were washed two additional times in order to ensure complete removal of Ca^{+2}. After the final wash, the cell pellet was resuspended in 1 ml of DPBS without Ca^{+2} and Mg^{+2}. Aliquots of approximately 100,000 cells were transferred to pre-chilled 1.5 ml tubes and centrifuged at 300 x g for 5 min at 4°C. The supernatant was removed, and the cell pellets were carefully resuspended in 488 µl of ice-cold DPBS without Ca^{+2} and Mg^{+2}. The tubes were placed in a 37°C water bath for 2 min before 12 µl of the SLO working solution was added to each tube. The tubes were then incubated horizontally in a 37°C water bath for 50 min. At the end of the incubation period, the tubes were placed on ice and 500 µl of ice-cold DPBS without Ca^{+2} and Mg^{+2} was added to each tube. Prior to extract exposure, the permeabilized BFF cells were centrifuged at 300 x g for 5 min at 4°C, and the supernatant removed.

In Vitro Reprogramming

An ATP-Regenerating System was prepared by mixing ATP (100 mM), phosphocreatine (1 M), creatine kinase (2.5 mg/ml), and GTP (10 mM) in a 1:1:1:1 ratio. A volume of 5 µl of the ATP-Regenerating System mix was added to each 100 µl aliquot of ADAS cell extracts and then transferred to the 1.5 ml tubes containing 100,000 permeabilized BFF cells. Controls for each BFF cell line were prepared by adding equal

volume of DPBS with Ca^{+2} and Mg^{+2} to 100,000 permeabilized cells. The cells were

incubated horizontally in a 37˚C water bath for 1 h. At the end of incubation, 500 µl of

complete culture medium containing 2 mM $CaCl_2$ (added from a 1 M $CaCl_2$ stock

solution) was added to each 1.5 ml tube to reseal the plasma membrane of the BFF

cells. The contents of each tube were then transferred to one Matrigel-coated well of a

24-well plate and cultured for approximately 4 h in a 5% CO_2 incubator at 37˚C before

the replacing the 2 mM $CaCl_2$-containing culture media with fresh complete culture

medium (without added $CaCl_2$). The cells were cultured in a 5% CO_2 incubator at 37˚C

until assessment of reprogramming efficiency.

Isolation of mRNA

Approximately 72 h after extract exposure, mRNA was isolated from both

treatment and control cells using Dynabeads® mRNA Direct™ Micro Kit (Dynal Biotech,

Inc., Lake Success, NY, USA) as described previously by Wrenzycli et al (2001). After

cells were harvested by trypsinization, they were washed in 1 ml of DPBS with Ca^{+2} and

Mg^{+2} and 300 µl of lysis/binding buffer (100 mM Tris HCl (pH 8.0), 500 mM LiCl, 10 mM

EDTA, 1% lithium dodecylsulfate, and 5 mM dithiothreitol). DNA was sheared from the

cells using a 1 ml syringe and a 21 gauge needle before being centrifuged at 12,000 x g

for 15 sec. Following a 10 min incubation, pre-washed Dynabeads® (50 µl) were added

to the sample solute, and the sample poly(A)+ RNAs were allowed to anneal to the

beads while rotating on a hybridization mixer for 10 min. The beads were separated

from the mix using a Dynal MPC-E-1 magnetic separator. The samples were washed

twice in 50 µl of wash buffer A (10 mM Tris HCl (pH 8.0), 150 mM LiCl, 1 mM EDTA,

and 0.1% lithium dodecylsulfate) and twice in 50 µl wash buffer B (10 mM Tris HCl (pH

8.0), 150 mM LiCl, and 1 mM EDTA). The mRNA was eluted from the beads by adding

15 µl of nuclease-free water and incubating for 2 min in a 70°C water bath. The mRNA

was separated from the beads using the Dynal MPC-E-1 magnetic separator and

immediately used for reverse transcription.

Reverse Transcription

The freshly-isolated mRNA was reverse transcribed into cDNA in a total volume

of 20 µl using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA,

USA). Each 20 µl iScript RT Reaction mix consisted of 15 µl of sample mRNA, 1 µl of

reverse transcriptase, and 4 µl of iScript reaction mix. The reaction was conducted at

25°C for 5 min, 42°C for 30 min, a denaturation step of 85°C for 5 min, and a final

holding temperature of 4°C.

Validation of Real Time PCR

To ensure that the primers for PAP, Oct-4, Nanog, and Sox-2 amplified a single

product in a quantitative manner, amplification efficiency and a correlation coefficient

from a standard curve was determined for each gene using qRT-PCR (Figure 3.1).

Purelink™ PCR Purification Kit (Invitrogen, K3100-01) was used to purify PCR products

in order to individually optimize each transcript in the amplified calibrator cDNA. A

standard curve for each gene was generated from the purified PCR product at six

different 10-fold dilutions. The calibrator cDNA was produced from a mixture of RNA

from two BFF cell lines at four different passages that was reverse transcribed into

cDNA using the same method previously described. All of the target genes had

acceptable efficiencies (80 to 120%) and correlation coefficients (close to 1.0).

RT-PCR followed by gel electrophoresis was first employed to confirm the presence of Oct-4, Nanog and Sox-2 transcripts in BFF cells treated with ADAS cell extracts. Primer sets for Oct-4, Nanog, and the reference gene, polyadenylate polymerase (PAP), have previously been verified to amplify their respective transcripts in bovine embryos. All primers were designed from bovine gene sequences using the Beacon Designer 4.0 (PREMIER Biosoft International) (Table 4.1), and were diluted to 10 mM concentration. Reactions were carried out in a total volume of 25 µl, which consisted of 2.5 µl cDNA, 1 µl of each primer (sense and antisense), 12.5 µl of JumpStart™ REDTaq® ReadyMix™ PCR Reaction Mix (Sigma, No. P0982), and 8 µl of water. The program used to amplify all genes in each sample involved a denaturating cycle of 1 min at 95°C; 35 cycles of PCR (95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec); 4 min at 72°C; and a final holding temperature of 4°C. PCR products were electrophoresed on a 2% agarose gel and sequenced to confirm the correct products were amplified. To assure that the primer sets not amplify genomic DNA, 1 ng of genomic DNA was used as a template for the amplification of the target genes. No amplicons were recovered after RT-PCR of genomic DNA (data not shown).

Quantitative Real-Time PCR

The expression levels of Oct-4, Nanog and Sox-2 in BFF cells treated with ADAS cell extracts were quantified using The LightCycler® 480 Real-Time PCR System (Roche Applied Science, Indianapolis, IN, USA). cDNA was amplified using SsoFast™ EvaGreen supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The total 20 µl real time PCR mix consisted of 2 µl of cDNA, 10 µl of SsoFast™ EvaGreen Supermix,

Table 4.1. Primers used for qRT-PCR analysis

Gene	Accession Number	Primers	Amplicon Length
Oct-4	NM174580	Sense GGTTCTCTTTGGAAAGGTGTTC Antisense ACACTCGGACCACGTCTTTC	223
Nanog	DQ069776	Forward AATTCCCAGCAGCAAATCAC Reverse CCCTTCCCTCAAATTGACAC	215
Sox-2	NM001105463	Sense AGGACTGAGAGAAAGAAGAAGAG Antisense AAGAAAGAGGCAAACTGGAATC	164
PAP	X63436	Sense AAGCAACTCCATCAACTACTG Antisense ACGGACTGGTCTTCATAGC	169

6 μl of nuclease-free water, and 1 μl of forward and reverse primer pairs (10 mM) for each gene. Within each qRT-PCR plate setup, reactions for the reference gene and each gene of interested were performed using the calibrator cDNA, the sample cDNA, and a no template negative control. The PCR program used for the amplification of all genes consisted of a denaturing cycle of 30 sec at 95°C; 45 cycles of PCR (95°C for 5 sec and 55°C for 20 sec); a melting curve analysis which consisted of 95°C for 5 sec, 65°C for 1 min, followed by continuous acquisition at 97°C, with 5 acquistions per °C; and a final holding temperature of 40°C.

Data was quantified using the method for relative quantification in qRT-PCR described by Pfaffl (2001). Values are reported as relative transcription or the n-fold difference relative to a calibrator. A mixture of cDNA from BFF cells at multiple passages was used as a calibrator for all of the target genes. PAP was used as the internal reference gene. The threshold cycle (Puissant et al.) value of the referencegene was used to normalize the target gene signals in each sample. The amount oftarget transcripts relative to the calibrator was calculated using the following equation: n-fold difference = Efficiency Target Gene$^{\Delta C_T T}$/ Efficiency Reference Gene$^{\Delta C_T R}$, where an efficiency value of two was assumed. The $\Delta C_T T$ (for the target gene) value was calculated by subtracting the sample C_T value of the target gene from the calibrator C_T value of the target gene. The $\Delta C_T R$ (for the reference gene) value was calculated by subtracting the sample C_T value of the reference gene PAP from the calibrator C_T value of the reference gene. Therefore, all target abundance levels were expressed as n-fold differences relative to a calibrator and normalized to the reference gene in order to compensate for PCR variations between runs.

Statistical Analysis

Data were analyzed using SigmaStat Statistical Software Version 3.5 (Systat Software, Richmond, CA, USA). A T-test was performed to compare Oct-4, Nanog and Sox-2 expression levels between all untreated ADAS cell and BFF cell samples with BFF cells exposed to ADAS cell extracts and BFF cells exposed to hESC extracts. Differences of $P < 0.05$ were considered to be significant.

A 95% confidence interval for the gene expression levels for each gene of interest was formulated using descriptive statistics. If the n-fold difference relative to the calibrator for each treatment group equivalent did not fall within the confidence interval for the untreated group, they were considered abnormal, either upregulated or downregulated.

Results

The extract-based nuclear reprogramming procedure was performed a total of nine times; however, only four of these times did BFF cells survive in culture following treatment. Initially, we exposed permeabilized BFF cells to extracts that had previously been prepared and stored at -80°C. When we observed that none of these cells survived, we attributed it to instability of the extracts as a result of being stored at -80°C for an extended period of time. While Collas's (www.collaslab.com) protocol for extract-based reprogramming of somatic cells states that extracts can be frozen and stored at -80°C, it does recommend carrying out reprogramming reactions with freshly prepared extracts as the stability of extracts stored and frozen at -80°C may vary with cell types and batches. Based on this, we carried out all subsequent reprogramming reactions with freshly prepared extracts; however, this did not ensure cell survival following every

treatment. Of the eight additional times we conducted the reprogramming experiment, only four times did the treated cells survive in culture.

The values corresponding to the relative expression of Oct-4, Nanog and Sox-2 in all BFF cells exposed to ADAS cell extracts and hESC extracts are reported in tables 4.2.and 4.3. Statistical analysis comparing Oct-4, Nanog and Sox-2 expression levels in all untreated ADAS cells and BFF cell samples at all three passages with those of BFF cells exposed to ADAS cell extracts revealed no significant difference in Oct-4, Nanog and Sox-2 expression. Comparisons between only untreated ADAS samples and BFF cells exposed to ADAS cell extracts also revealed no significant difference in Oct-4, Nanog and Sox-2 expression levels. Interestingly, a significant difference in Sox-2 expression between untreated BFF cells and BFF cells exposed to ADAS cell extracts was found (P = 0.003); however, there was no significant difference in either Oct-4 or Nanog expression in these two groups (Table 4.4).

Statistical analysis comparing Oct-4, Nanog and Sox-2 transcript levels in all untreated ADAS cells and BFF cell samples at all three passages with those of BFF cells exposed to hESC extracts revealed no significant difference in Oct-4, Nanog and Sox-2 expression. Likewise, no significant difference in Oct-4, Nanog and Sox-2 expression was found when only untreated ADAS cell samples were compared to BFF cells exposed to hESC extracts and when only untreated BFF cell samples were compared to BFF cells exposed to hESC extracts (Table 4.5).

Table 4.2. Oct-4, Nanog and Sox-2 relative expression levels in BFF cells treated with ADAS cell extracts[1]

Sample	Oct-4	Nanog	Sox-2
BFF#1 w/ A (Rep 1)	5.90	0.24	0.60
BFF#1 w/ A (Rep 2)	0.78	0.18	0.61
BFF#1 w/ A (Rep 3)	0.84	0.19	0.32
BFF#1 w/ B	1.40	$2.43E^{-2}$	1.10
BFF#1 w/ D	0.03	6.33	0.45
BFF#2 w/ B	$2.55E^{4}$	$4.61E^{-2}$	1.13
BFF#2 w/ C	0.05	23.09	0.23
BFF#2 w/ D	0.07	12.72	0.21

[1] BFF#1 and BFF#2 were treated with extracts derived from four ADAS cell lines, designated A, B, C and D

Table 4.3. Oct-4, Nanog and Sox-2 relative expression levels in BFF cells treated with hESC extracts

Sample	Oct-4	Nanog	Sox-2
BFF#1 (Rep 1)	$4.60E^{-3}$	$3.81E^{-5}$	$3.47E^{-4}$
BFF#1 (Rep 2)	$6.24E^{-3}$	$4.14E^{-5}$	$2.35E^{-4}$
BFF#2 (Rep 1)	$1.64E^{-2}$	$4.97E^{-2}$	$5.83E^{-3}$
BFF#2 (Rep 2)	4.13	3.35	2.70
BFF#2 (Rep 3)	$5.04E^{-2}$	$2.44E^{-3}$	$3.20E^{-4}$
BFF#2 (Rep 4)	$9.94E^{-2}$	$3.03E^{-3}$	$1.56E^{-3}$

Table 4.4 Comparison of the mean Oct-4, Nanog and Sox-2 expression levels between untreated BFF cells and BFF cells treated with ADAS cell extracts

Gene	Untreated BFF n=6	BFF w/ ADAS extracts n=8
Oct-4	0.39	3188.63
Nanog	4.19	5.35
Sox-2	0.03[a]	0.58[a]

Statistical differences were determined by t-test (P < 0.05)
Significant differences represent by [a] (P = 0.003)

Table 4.5 Comparison of the mean Oct-4, Nanog and Sox-2 expression levels between untreated BFF cells and BFF cells treated with hESC extracts

Gene	Untreated BFF n=6	BFF w/ hESC extracts n=6
Oct-4	0.39	0.72
Nanog	4.19	0.57
Sox-2	0.03	0.45

Statistical differences were determined by t-test (P < 0.05)
No significant differences between treatments

Discussion

The remarkable ability to give rise to all the cell types of the body not only defines the inherent nature of ESC, but also underlines their enormous potential as donor cells for cell transplantation therapies. Despite the promise of ESC, ethical concerns and potential immune rejection after transplantation are current major hindrances of their use. Reprogramming the nuclei of differentiated cells to an ESC-like state may be a solution to both of these problems. Successful reprogramming of somatic cells towards pluripotency has been accomplished through SCNT (Wilmut et al., 1997), fusion with ESC (Cowan et al., 2005), ectopic expression of defined transcription factors (Takahashi and Yamanaka, 2006), and exposure to ESC extracts (Bru et al., 2008). Each of these methods has proven to facilitate the reacquisition of pluripotency in differentiated somatic cell nuclei, yet no technique is without its limitations. Nonetheless, these four different experimental strategies demonstrate that pluripotency can be restored in terminally differentiated cells, proving that the epigenetic state of somatic cells is not irreversibly fixed (Jaenisch and Young, 2008).

In the present study, we detected the presence of Oct-4, Nanog and Sox-2 transcripts in both bovine ADAS cells and BFF cells. The presence of these central transcription factors characteristic of pluripotent stem cells suggests that these cells may possess characteristics similar to ESC, including epigenomic regulatory pattern. Therefore, ASC may be better equipped for complete epigenetic reprogramming than terminally differentiated cells. Because the endogenous expression of Oct-4, Nanog and Sox-2 is believed to contribute towards reprogramming efficiency, we sought to increase their expression level in BFF cells through exposure to nuclear and

cytoplasmic extracts derived from ADAS cells. Extract-based reprogramming

approaches have shown that differentiated cells may be induced to transdifferentiate

into other differentiated cell types (Collas and Håkelien, 2003) or dedifferentiate towards

pluripotency (Taranger et al., 2005; Bru et al., 2008). To our knowledge, this is the first

report measuring Oct-4, Nanog and Sox-2 expression levels in ASC exposed to extracts

of other ASC.

The successful reprogramming of cells using the cell-extract based system has

been reported by many groups (Ha°kelien et al., 2002; Landsverk et al., 2002; Collas

and Håkelien, 2003; Taranger et al., 2005; Freberg et al., 2007; Bru et al., 2008);

however, we observed a great deal of variability in cell survival between experiments.

Such variability in the effectiveness of reprogramming attempts using cell extracts has

also been observed by others (www.collaslab.com). This variability may be due to

differences in the relative reprogramming potential of ADAS cell extracts prepared, or

the effectiveness of reversible permeabilization, which was not monitored all ten times

the experiment was conducted. Furthermore, cell extracts have been reported to

manifest varying amounts of toxicity, with some extracts being completely ineffective for

reprogramming (Ha°kelien et al., 2002).

Even in the four "successful" experiments, we observed many dead cells in

culture following treatment. Fortunately, in these cases enough cells survived treatment

to expand in culture, thus allowing mRNA to be isolated. It should be noted that we only

had to conduct the reprogramming experiment with hESC once, as the BFF survived

exposure to these extracts. While we cannot rule out that this is due merely to chance,

it may be that extracts derived from hESC are less toxic to permeabilized cells than

extracts derived from somatic cells. This is simply an observation, and validation of this hypothesis has not been reported.

In other studies, successful reprogramming was marked by factors such as the nuclear uptake of transcription factors and cell surface antigens specific to the cell type from which the extracts were derived, chromatin remodeling activity, changes in gene expression patterns, and changes in morphology. In most of these studies, the cell type being reprogrammed was much different than that of the cell type from which the reprogramming extracts were derived; therefore, factors such as gene induction were measurable. Because Oct-4, Nanog and Sox-2 were found to be present in both BFF cells and ADAS cells, we could not assess induced expression of otherwise repressed genes. Instead, we analyzed samples for changes in the expression pattern of these genes. Unlike other studies that have reported the upregulation of Oct-4, Nanog and Sox-2 in cells treated with extracts derived from hESC (Taranger et al., 2005; Freberg et al., 2007) and murine ESC (Bru et al., 2008), our statistical analysis revealed no significant difference in Oct-4, Nanog and Sox-2 expression levels between untreated BFF cells and ADAS cells and BFF cells treated with hESC. One possible explanation for this may be due to expression differences between species. Another explanation for this may be the use of reprogramming extracts derived from higher passage number ESC as opposed to very early passage number ESC. The hESC we used to derive nuclear and cytoplasmic extracts had previously been cultured for an unknown number of passages before being frozen and then given to us for culture. Furthermore, after the initial plating on feeder layers of MEF, we passaged the hESC two additional times before deriving extracts from them. Although we do not know the exact passage

number of the hESC when we received them, it is likely that by the time we obtained extracts from these cells they were at a high passage number.

In the present study, we did not detect a significant difference in Oct-4 and Nanog transcript levels between untreated BFF cells and ADAS cells and BFF cells exposed to ADAS cell extracts. However, a comparison between untreated BFF cells and BFF cells treated with ADAS cell extracts revealed a significant difference in Sox-2 expression levels. It should be noted that Sox-2 expression levels were not found to be significantly different between untreated ADAS cells and BFF cells treated with ADAS cell extracts. This was also the case when we compared all untreated BFF cells and ADAS cells with BFF cells exposed to ADAS cell extracts. It is surprising that Sox-2 expression levels increased significantly following treatment, but Oct-4 and Nanog levels did not as it has been shown that the expression levels of these three transcriptional regulators are tightly linked (Boyer et al., 2005). Other studies have reported similar findings, in which an increase in Oct-4 expression was not accompanied by an increase in Nanog expression following extract exposure (Neri et al., 2007). The investigators in these studies also reported being surprised by their findings, and concluded that in these instances only partial reprogramming of the donor cell nucleus occurred. It is possible that our findings are due to only the partial reprogramming of BFF nuclei following extract exposure. Because Oct-4 and Nanog expression is repressed before Sox-2 during differentiation, it may be more difficult to reprogram Oct-4 and Nanog to a sufficient state. However, further research is needed before a definitive conclusion can be made regarding the pattern of gene expression we noted in our study.

CHAPTER V
SUMMARY AND CONCLUSIONS

The extraordinary nature of ESC lends them the remarkable ability to give rise to all the cell types of a mammalian organism, an attribute known as pluripotency. The pluripotent state of ESC is dependent upon the expression of the genes Oct-4, Nanog and Sox-2, which have also been identified as the key transcriptional regulators of pluripotency (Boyer et al., 2005). Until recently, Oct-4, Nanog and Sox-2 were believed to be expressed solely in ESC; however, studies have reported the expression of these genes in some sources of ASC of both the mouse and pig (Kues et al., 2005; Carlin et al., 2006). Furthermore, these ASC as well as several other sources of ASC have been shown to exhibit a surprisingly high degree of plasticity. This remarkable feature allows ASC to cross lineage barriers and adopt the expression profiles and functional phenotypes of cells unique to other tissues (Herzog et al., 2003). These findings suggest that ASC possess characteristics similar to pluripotent ESC, including epigenetic regulatory pattern. Considering the abundance and accessibility of adipose tissue, ADAS cells are an attractive source of stem cells for use in research and biomedical applications.

In the present study, we first examined cells derived from bovine adipose tissue as well as fetal fibroblasts for the expression of Oct-4, Nanog and Sox-2. Transcripts for all three pluripotency-associated genes were detected in all BFF cell and ADAS cell samples at every passage analyzed. It has been suggested that the genome of less differentiated cells may be more amenable to reprogramming or require less reprogramming following the induction of pluripotency either by SCNT or another

experimental strategy (Rideout et al., 2001). Because of the high degree of plasticity demonstrated by ADAS cells, it is likely that these cells have an epigenomic regulatory pattern that is closer to pluripotent ESC than terminally differentiated somatic cells (Sun et al., 2009). Although we found the Oct-4, Nanog and Sox-2 expression levels in ADAS cell samples to be quite low and highly variable between cell lines and passage numbers, their presence supports the notion that these cells are less differentiated than other somatic cells.

Because the endogenous expression of Oct-4, Nanog and Sox-2 is believed to contribute to reprogramming efficiency, we next attempted to increase the expression levels of these genes by exposing BFF cells to ADAS cell extracts. Extract-based reprogramming approaches have shown that differentiated cells may be induced to transdifferentiate into other differentiated cell types (Collas and Håkelien, 2003) or dedifferentiate towards pluripotency (Taranger et al., 2005; Bru et al., 2008). Because the genome of ASC is inherently less differentiated than other somatic cells, exposing them to extracts of other ASC may drive them further towards an undifferentiated state. Extract-based reprogramming using ASC is a promising and plausible approach towards the production of replacement cells for therapeutic purposes, as well as, the production of suitable donor cells for SCNT.

As with any technology, there is always room for improvement, and extract-based reprogramming of somatic cells is no exception. The degree of variability in the effectiveness of reprogramming attempts using cell extracts is, in our opinion, this reprogramming strategy's greatest hindrance. Furthermore, the application of this technology to produce replacement cells for therapeutic purposes requires significant

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developments and a large body of data is needed before this system can be applied to

the generation of cells used for therapy in human patients (Collas and Håkelien, 2003).

We believed that the high variability in Oct-4, Nanog and Sox-2 expression we observed

in ADAS cells makes their response to extract treatment unpredictable.

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APPENDIX A: PROTOCOLS

ADAS AND BFF CELL ISOLATION PROTOCOL

1. Collect adipose tissue from each specimen and place in the wash solution. Maintain samples on ice

2. Under a hood, transfer tissue to a weighing boat and mince the tissue

3. Transfer minced tissue to a 50 ml conical tube and add 25 ml of wash solution

4. Shake tube vigorously for 30 sec and allow phases to separate (about 5 min)

5. Carefully aspirate solution so not to remove any of the sample

6. Repeat wash, shake, and aspiration

7. Pour sample into a 150 ml Erlenmeyer flask. (if sample does not easily pour into the flask, remove using sterile forceps)

8. Add approximately 15-20 ml of .25% collagenase solution (.25% collagenase + 1% BSA (Fraction V + PBS with Ca^{+2} and Mg^{+2})

9. Add 200 μL of P/S and 200 μL of Fungizone to the flask containing tissue and collagenase solution

10. Cover flask with parafilm and place in shake incubator set at 200 rpm at 37°C for 2 h

11. After incubation, transfer entire suspension to a new 50 mL tube

12. Centrifuge 300 x g for 5 min

13. Following centrifugation, shake tubes vigorously for 15 to 30 sec

14. Repeat centrifugation

15. Remove tissue from flask, leaving liquid in the tube and shake

16. Pour contents into a double filter syringe system with a new 50 ml tube receiving the filtered solution

17. Centrifuge 300 x g for 5 min

18. Aspirate collagenase solution

19. Resuspend pellet in 15 ml of PBS with Ca^{+2} and Mg^{+2} + 1% BSA (Fraction V)

20. Centrifuge 300 x g for 5 min

21. Aspirate supernatant

22. Add approximately 10 ml of DMEM + 10% FBS + 1% P/S + 2% Fungizone

23. Centrifuge 300 x g for 5 min

24. Aspirate supernatant

25. Resuspend cell pellet in 1 ml of complete culture media (BFF cells: DMEM with 15% FBS + 1% P/S; ADAS cells: DMEM with 10% FBS + 1% P/S) and plate in appropriate size flask

26. Add appropriate amount of complete culture media to flask and place in incubator

27. After 24 h, remove flask from incubator and perform a primary wash.

TRI REAGENT® RNA ISOLATION PROTOCOL

1. Remove media from culture dish

2. Add 1 ml of TRI Reagent® per 10cm^2 to culture dish and pipette up and down several times

3. Incubate homogenate at room temperature for 5 min

4. While homogenate is incubating, prep phase lock tubes by centrifuging 3500 x g for 10 min at 4°C.

5. Transfer TRI Reagent® cell suspension to Phase-Lock Gel tube

6. Add 0.1 ml of BCP per 1 ml of TRI Reagent used to suspension

7. Secure caps and shake vigorously for 30 sec

8. Incubate tube at room temperature for 1 h

9. After incubation period, centrifuge at 12,000 x g for 15 min at 4°C.

10. Transfer the clear aqueous supernatant to a fresh 15 ml tube

11. Precipitate RNA by adding 0.5 ml of isopropyl alcohol per 1 ml of TRI Reagent® used in initial homogenization to aqueous supernatant.

12. Store overnight at -20°C for optimum recovery of RNA

13. Centrifuge at 12000 x g for 20 min at 4°C. The RNA will form a small gel-like or white pellet.

14. Pour off supernatant away from the pellet

15. Centrifuge pellet at 12000 x g for 2 min at 4°C

16. Wash the RNA pellet in 75% EtOH (prepared in DEPC water) using at least 1 ml of EtOH per 1mL of TRI Reagent®

17. Vortex tube until pellet is floating

18. Centrifuge 14000 x g for 10 min

19. Carefully transfer the RNA pellet to a 1.5 ml tube containing 1 ml of 75% EtOH

20. Repeat centrifugation

21. Remove supernatant and allow pellet to dry under a laminar flow hood

22. Dissolve the RNA pellet in 50 µl of DEPC water and store at -80˚C

DYNABEADS[®] RNA ISOLATION PROTOCOL

1. Harvest cells from culture flask as per standard procedure

2. Wash cell pellet first in 1 ml of PBS with Ca^{+2} and Mg^{+2} and then in 300 µl of lysis/binding buffer (100mM Tris HCl (pH 8.0), 500 mM LiCl, 10 mM EDTA, 1% lithium dodecylsulfate, and 5 mM dithiothreitol)

3. Strip cells using a 21 gauge needle and a 1 ml syringe. Vortex for 10 sec.

4. Centrifuge at 12000 x g for 15 sec. and incubate at RT for 10 min.

5. Add 50 µl of the pre-washed oligo dT Dynabeads (dT_{25}) to the tube (pre-washed Dynabeads in lysis/binding buffer)

6. Incubate at RT for 10 min in hybridization mixer

7. Place tube in magnetic separator for 2 min

8. Remove supernatant and wash beads twice in 50 µl of Buffer A (10 mM Tris HCl (pH 8.0), 150 mM LiCl, 1 mM EDTA, 0.1% lithium dodecylsulfate) and twice in 50 µl of Buffer B (10 mM Tris HCl (pH 8.0), 150 mM LiCl, 1 mM EDTA).

9. Elute RNA from the beads by adding 15µL of nuclease-free water and heating the sample at 70˚C for 2 min.

10. Use sample directly for reverse transcription

cDNA SYNTHESIS PROTOCOL

1. Mix 4 µl of iScript reaction mix, 1 µl of reverse transcriptase, and 15 µl of sample

    mRNA in PCR tube (Bio-Rad Laboratories, Inc., Hercules, CA, USA)

2. Place tubes in the thermocycler and run for 5 min at 25˚C, 30 min at 42˚C,

    denaturation at 85˚C for 5 min, and a final hold at 4˚C.

RT-PCR PROTOCOL

1. Each reaction is carried out in a total of 25 µl. Mix 12.5µL of JumpStart™

    REDTaq$^®$ ReadyMix™ PCR Reaction Mix (Sigma, No. P0982), 8 µl of water,

    1 µl of each [10mM] primer (sense and antisense), and 2.5 µl of sample cDNA.

    Prepare master mixes whenever possible.

2. Place tubes in thermocycler and run a denaturating cycle of 1 min at 95˚C; 35

    cycles of PCR (95˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 30 sec); 4 min at

    72˚C; and a final holding temperature of 4˚C.

3. Upon completion, remove samples from the thermocycler and add 12.5 µl of

    PCR product to a 2% agarose gel for gel electrophoresis.

qRT-PCR PROTOCOL

1. Prepare master mixes for each gene being analyzed (Oct-4, Nanog, Sox-2, PAP). Each reaction contains 10 µl of SsoFast™ EvaGreen supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 6 µl of nuclease-free water, 1 ul of each [10 mM] primer (sense and antisense), and 2 µl of either sample or calibrator cDNA (added later).

2. Pipette 18 µl of the appropriate master mix into the designated wells of a 96 multiwell plate tailor-made for LightCycler® 480 Instrument (Roche No. 04729692001).

3. Add 2 µl of either sample or calibrator cDNA to each designated well

4. Cover plate with sealing foil

5. Place plate in the LightCycler® 480 Instrument and run a denaturing cycle of 30 sec at 95°C; 45 cycles of PCR (95°C for 5 sec and 55°C for 20 sec); a melting curve analysis which consisted of 95°C for 5 sec, 65°C for 1 min, followed by continuous acquisition at 97°C, with 5 acquistions per °C; and a final holding temperature of 40°C.

REPROGRAMMING SOMATIC CELLS IN CELL-FREE EXTRACTS PROTOCOL

Derivation of Extracts

1. Remove cells from culture and remove media

2. Wash with PBS without Ca^{+2} and Mg^{+2} and remove

3. Add appropriate amount of Trypsin to flask (.5 ml for T-25)

4. Place in 37°C oven for about 5 min

5. Add approximately 5 ml of complete culture medium and move to 15 ml tube

6. Centrifuge at 300 x g for 10 min at 4°C

7. Remove remaining media

8. Wash cells in 10 ml of ice cold PBS with Ca^{+2} and Mg^{+2} and centrifuge at 300 x g for 10 min at 4°C. Discard supernatant.

9. Repeat

10. Remove 500 µl of Cell Lysis Buffer (without Protease Inhibitor Cocktail) and set aside. Resuspend cells in remaining ice-cold Cell Lysis Buffer (without Protease Inhibitor Cocktail)

11. Centrifuge at 300 x g for 10 min at 4°C. Discard supernatant.

12. Add 10 µl of Protease Inhibitor Cocktail and 5 µl of EDTA to the 500 µl of Cell Lysis Buffer.

13. Estimate cell pellet volume, and resuspend cells in 1 volume of Cell Lysis Buffer containing the Protease Inhibitor Cocktail and EDTA. For cells grown in T-12.5, add 250 µl of the 500 µl solution of Cell Lysis Buffer. Adjust as needed.

14. Transfer the cell suspensions to individual 1.5 ml tubes

15. Place tubes on ice for 30-45 min., tapping the tubes occasionally to keep cells suspended.

16. Remove cell suspension from each tube and move to pestle for Dounce Homogenization. Lyse cells on ice using the mortar and pestle. Monitor cell lysis progress using microscopy, and continue Dounce Homogenization until 90% of cells lysed.

17. Move cell lysate to pre-chilled 1.5 ml tubes. Make sure to get the liquid off of the sides and top of mortar and pestle.

18. Centrifuge these tubes containing cell lysate at 15,000 x g for 15 min at 4°C.

19. Carefully collect the supernatant from the tubes using a 200 μl pipette and transfer to a new pre-chilled 1.5 ml tube. The supernatant contains the extract and the pellet is discarded.

20. Resuspend the remaining extract using a 200 μl pipette and then aliquot 100 μl of extract into 1.5 ml tubes on ice.

21. At this point, extracts can be flash frozen using liquid N_2 and stored in -80°C till use; however, it is best to carry out reprogramming reactions with fresh extracts.

Permeabilization of Donor Cells

1. Remove aliquots of SLO in 10mM DTT from -20°C. (SLO powder dissolved in 10mM DTT and aliquot. Aliquots should be discarded after 1 month).

2. Dilute SLO stock in PBS without Ca^{+2} and Mg^{+2} in a 1:20 ratio. (The appropriate ratio for the given cell type indicates the proper concentration of SLO needed to cause 70-80% permeabilization). This is your "SLO working solution".

3. Keep "SLO working solution" on ice.

4. In the interim, remove media from "donor cells"

5. Wash cells in PBS without Ca^{+2} and Mg^{+2} and remove

6. Add appropriate amount of Trypsin to flask (.5 ml for T-25)

7. Place in 37°C oven for about 5 min

8. Add approximately 5mL of complete culture medium and move to 15mL tube

9. Centrifuge at 300 x g for 10 min at 4°C

10. Remove remaining media

11. Wash cells in ice-cold PBS without Ca^{+2} and Mg^{+2} and centrifuge at 400 x g for 10 min at 4°C. Discard supernatant and repeat this two more time. It is essential to remove all of the Ca^{+2} because it inhibits SLO activity.

12. After first wash in PBS without Ca^{+2} and Mg^{+2}, remove 10 µl of cell suspension and add to 90 µl of complete culture medium. Count cells.

13. When final centrifugation is complete, remove supernatant and resuspend cell pellet in 1 ml of PBS without Ca^{+2} and Mg^{+2} and aliquot desired number of cells (100,000) into 1.5 ml pre-chilled tubes.

14. Centrifuge these tubes at 300 x g for 5 min at 4°C. Discard the supernatant.

15. Carefully resuspend the cell pellet in 488µL of ice-cold PBS without Ca^{+2} and Mg^{+2} using a 1000 µl pipette with a large tip.

16. Place the tubes in 37°C water bath for 2 min and then add 12 µl of "SLO working solution". Mix by tapping on the tube.

17. Remove the tubes from the water bath and incubate horizontally at 37°C for 50 min. Tap occasionally on the tubes to maintain cell suspension.

18. After incubation, place tubes on ice and add 500 µl of ice-cold PBS without Ca^{+2} and Mg^{+2}

19. Centrifuge the tubes at 300 x g for 5 min at 4°C. Remove supernatant before in vitro reprogramming.

Cell Permeabilization Assay

1. Harvest donor cells as per standard protocol (see above)

2. Discard remaining media following centrifugation, and wash cell pellet in ice-cold PBS with Ca^{+2} and Mg^{+2}

3. Centrifuge at 300 x g for 10 min at 4°C

4. Discard supernatant and repeat wash step two more times. Do not discard supernatant after final spin.

5. Determine the number of cells you want to use (Collas suggests 500,000) and resuspend the cell pellet in the remaining PBS without Ca^{+2} and Mg^{+2} Aliquot desired number of cells into 1.5 ml tubes on ice

6. Centrifuge the 1.5 ml tubes at 300 x g for 5 min at 4°C. Discard supernatant.

7. Resuspend cell pellet in 488 µl of PBS without Ca^{+2} and Mg^{+2} that contains approximately 5 µl of Propidium Iodide

8. Place tubes in 37°C water bath for 2 min to allow cells to heat up

9. Next, add 12 µl of the ice-cold SLO working solution to each tube and mix by gentle pipetting

10. Incubate the tubes at 37°C for 50 min, tapping cells occasionally

11. Following incubation, centrifuge cells at 300 x g for 5 min at 4°C

12. Discard supernatant

13. Add 1.5 ml of complete culture medium, containing 2 mM of $CaCl_2$ to each tube

14. After a few minutes, observe cells under fluorescent microscopy to determine if permeabilization occurs and the cells took up the P.I. If the cells glow red, they took up P.I. P.I. stains DNA, so it is best to see the red concentrated in the nucleus. Calculate the percentage of cells that took up P.I. to determine the proper concentration of SLO. Optimal SLO concentration is indicated by approximately 70-80% of cells being permeabilized.

In Vitro Reprogramming

1. Make the ATP-Regenerating System mix at 1:1:1:1 ratio and keep on ice (Appendix B)

2. If extracts were not previously aliquoted, aliquot 100 µl of extracts into 1.5 ml tubes and hold on ice. If extracts were frozen, promptly thaw the extract between fingers and place on ice.

3. Add 5 µl of ATP-Regenerating System to 100 µl of extracts (adjust accordingly) and mix by gentle pipetting

4. Add extract and ATP-Regenerating System mix to tube of permeabilized cells. (For 100,000 cells, add 100µL of the mix. Adjust accordingly). Carefully suspend with 1000 µl pipette tip

5. Incubate cells horizontally in 37°C water bath for 1 h, tapping tubes occasionally.

6. At the end of the incubation, add 1 ml of complete culture medium containing 2 mM $CaCl_2$ added from 1 M stock to reseal the cell membrane.

7. Plate the contents of each tube and culture for 2 to 4 hours in 5% CO_2 incubator at 37°C (for 100,000 cells, plate in individual wells of 24-well plate).

8. After culture period, remove the dead cells (floating) and the $CaCl_2$ – containing media. Replace with the complete culture medium (without $CaCl_2$) that the cells are now being cultured in

9. Place cells back in the incubator until RNA isolation

APPENDIX B: REAGENT FORMULATIONS AND STOCK SOLUTIONS

Cell Lysis Buffer[1]

Component	Product Number	Company	Amount
100 mM HEPES	H3375	Sigma	1 ml (1 M)
50 mM NaCl	S5886	Sigma	1 ml (500 mM)
5 mM MgCl$_2$	M2393	Sigma	1 ml (50 mM)
0.1 mM Phenylmethysufonyl fluoride (PMSF)	P7626	Sigma	100 µl (10 mM)
1 mM Dithiothreitol (DTT)	D9779	Sigma	1 mM
Protease Inhibitor Cocktail (100X)	P2714	Sigma	10 µl per 500 µl of CLB
0.5 M EDTA	E5134	Sigma	5 µl per 500 µl of CLB

[1] Primary stocks of 1M HEPES, 500mM NaCl, 50mM MgCl$_2$ and 10mM PMSF were prepared and aliquots corresponding to their final concentration in the cell lysis buffer were made. For 1mM DTT, 1.54mg of DTT powder was added directly to HEPES, NaCl, MgCl$_2$ and PMSF, and the solution was brought up to 10 ml with sterile water. 10 µl of 100X Protease Inhibitor Cocktail and 5 µl of EDTA was added to a 500 µl aliquot of the cell lysis buffer.

ATP-Regenerating System Mix[2]

Component	Product Number	Company	Amount
100 mM Adenosine 5'-triphosphate disodium salt hydrate (ATP)	A3377	Sigma	1:1:1:1
10 mM Guanosine 5'-triphosphate sodium salt hydrate (GTP)	G8877	Sigma	1:1:1:1
1 M Phosphocreatine	P7936	Sigma	1:1:1:1
2.5 mg/ml Creatine Kinase	C3755	Sigma	1:1:1:1

[2] Primary stocks of 100 mM ATP, 10 mM GTP, 1 M Phosphocreatine and 2.5 mg/ml Creatine Kinase were prepared, aliquoted and stored at -20° C. The ATP-Regenerating System Mix was prepared fresh by mixing ATP, GTP, Phosphocreatine and Creatine Kinase in a 1:1:1:1 ratio from each separate stock. For every 100 μl of extract, a total of 3 μl of ATP-Regenerating System Mix was supplemented.

VITA

Laura Whitney Coley was born on March 11, 1985 to Howard and Ann Coley in Texarkana, Texas. In 2003, she graduated from Arkansas High School in Texarkana, Arkansas.

Following high school, Laura moved to Baton Rouge, Louisiana to pursue a Bachelor of Science degree in animal, dairy and poultry sciences from Louisiana State University. During her undergraduate studies, Laura became interested in reproductive physiology, and in her senior year, she participated in an undergraduate research project under the supervision of Dr. Kenneth R. Bondioli and Dr. Robert A. Godke.

Laura entered the graduate program in reproductive physiology under the direction of Dr. Kenneth R. Bondioi in the fall of 2008 and is now a candidate for the degree of Master of Science in reproductive physiology in the Department of Animal Sciences at Louisiana State University, Baton Rouge, Louisiana.