2012

Lentiviral transduction of epigenetically modified bovine adult stem cells

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LENTIVIRAL TRANSDUCTION OF EPIGENETICALLY MODIFIED BOVINE ADULT STEM 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
Meredith Kathleen Addison
B.S., Louisiana State University, 2009
August 2012

ACKNOWLEDGEMENTS

The author would like to first acknowledge Dr. Kenneth Bondioli. Dr. Bondioli was kind enough to answer his office door in May 2009, along with the many questions that followed to a student determined to become part of his embryology program. Dr. Bondioli provided the author with endless guidance, encouragement, and patience while she pursued her degree. The author extends her deepest gratitude to Dr. Bondioli.

Next, the author would like to thank Dr. Glen Gentry for his contribution to a stimulating and healthy work environment. While director of the Reproductive Biology Center, Dr. Gentry provided the author with continual support and guidance. The author would also like to thank Dr. Kenneth Eilertsen at Pennington Biomedical Research Center for being a part of her graduate committee along with his advice and reassurance while writing her thesis.

The author would next like to thank Laura Whitney Coley for her immense help and strong friendship. Without Whitney's continuous help, friendship, and proof-readings, this thesis would not have been completed. The author is genuinely thankful for Whitney "taking her under her wing", and for the amazing friendship that she has gained from the experience.

The author would like to thank her fellow graduate students for providing the best environment of motivation, research, and friendship possible. Those students include Elliott Scroggs, Brittany Scott, Cody Bailey, Jaime Angulo, David Carwell, Jairo Sarmiento, and Tricia Font Adams. The author would also like to thank her best friends, Bailey Herring and Danielle Boudreaux, for their

encouragement, for being there when she needed a break, and for constantly offering a listening ear.

The author would not have been here was it not for her parents, Mark and Angie, and her brothers, John and Daniel. Mark and Angie Addison, along with Buddy and Pearl Ratcliff and Harry and Lil Addison provided the best atmosphere possible filled with immeasurable love and extraordinary inspiration which allowed the author to achieve the triumphs she has accomplished.

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ABSTRACT

Bovine adipose-derived stem cells (ADS), a form of adult stem cells, are somatic cells that have similar characteristics of embryonic stem (ES) cells. Bovine ADS cells possess multipotent capabilities and have been found to express pluripotency genes associated with ES cells. The unique properties of ADS cells make them a desirable source for reprogramming experiments. The goal of reprogramming experiments is to transform somatic cells from a differentiated state to a pluripotent state. When somatic cells reprogram, there are certain epigenetic changes or modifications that must occur in order to successfully reprogram the nucleus. Epigenetic modifications will change the chromatin configuration without changing the DNA sequence. Somatic cells can be exposed to small molecules that may be able to reduce the chances of having incomplete chromatin modification. Two epigenetic modifying factors are a DNA methyltranferase inhibitor, zebularine (Zeb), and a histone deacetylase inhibitor, valproic acid (VPA). By inducing gene expression with the epigenetic modifiers, the cells may be stimulated to reprogram more efficiently than cells with lower gene expression.

In the first experiment, three bovine ADS cell lines were treated with VPA or Zeb to observe the changes in expression levels of Oct4, Sox2, and Nanog (pluripotency-associated genes). The cells were treated for a period of 5, 7,10, or 14 days. VPA led to the highest increase of the pluripotency genes; however, both treatments may have produced a partial reprogramming. This partial

reprogramming may result in the bovine ADS cells reaching complete pluripotency when combined with a reprogramming technique.

In the second experiment, three bovine ADS cell lines were treated with VPA or Zeb for five days then followed with transduction using lentivirus. Oct4, Sox2, and Nanog were increased the highest when using epigenetic modifiers. Statistical differences for expression of the pluripotency-associated genes were found for cells treated with zebularine. While it was thought that viral transduction in combination with epigenetic modifiers would produce higher expression levels of the pluripotency-associated genes, this was not found to be true in this experiment.

CHAPTER I
INTRODUCTION

A growing field in today's clinical research is regenerative medicine. Regenerative medicine uses stem cells or pluripotent cells to regenerate and repair damaged tissue. Stem cell regenerative medicine can potentially reverse dysfunctional or damaged cells caused by disease or injury (Odorico et al., 2001). Stem cells are the ideal cell for regenerative medicine based on their distinctive qualities, and the cells are able to constantly regenerate, while maintaining the capability to differentiate into multiple cell lineages when induced in culture (Bunnell et al., 2008). There are two main types of stem cells used for this purpose, embryonic stem (ES) cells and adult stem cells. ES cells originate from the inner cell mass of blastocysts (Martin, 1981), and they are able to form the three germ layers of the embryo (endoderm, mesoderm, and ectoderm) (Evans and Kaufman, 1981). Because ES cells are difficult to isolate and harvest, recipient rejection, contamination, and tumor production, adult stem cells have been considered. Some sources of adult stem cells are adipose tissue, cartilage, and bone marrow. Adipose stem cells have been isolated and characterized (Zuk et al., 2002; Gimble and Guilak, 2003; Bunnel et al., 2008), and like adipose stem cells in humans, bovine adipose stem cells were found to differentiate into chondrocytes, osteoblasts, and adipocytes (Picou, 2009). The experiments conducted in this study, focus on producing less differentiated or pluripotent cells to use as donors for nuclear transfer (NT). We hope to further modify adult stem cells to make them more readily reprogrammable by NT.

In the first experiment, bovine adipose-derived stem (ADS) cells were treated with epigenetic modifiers and observed for changes in expression levels of pluripotency-associated genes. We hypothesize that these ADS cells could be reprogrammed to a more pluripotent state. If somatic cells contained pluripotent qualities, they could potentially differentiate into cells of the three germ layers that generate an embryo or certain cell types. Three lines of bovine ADS cells were treated with valproic acid (VPA), a histone deacetylase inhibitor, or zebularine (Zeb), a DNA methyltransferase I inhibitor, for 5, 7, 10, or 14 days at which point quantitative RT-PCR was conducted.

In the second experiment, bovine ADS cells were treated with epigenetic modifiers for 5 days before being infected with a lentivirus containing transcription factors for Oct4, Sox2, Klf4, and c-Myc. Cells were cultured for 14-16 days for colony growth before being passaged a second time once colony formation was detected. For both experiments, morphology and expression of Oct4, Sox2, and Nanog gene transcripts was observed. Our hypothesis was that with pretreatment of cells with epigenetic modifiers, transduction of bovine ADS cells with lentiviral particles would produce a more pluripotent cell.

CHAPTER II
LITERATURE REVIEW

Stem Cells

Over the years, interest in stem cells has intensified due to the cells' unique characteristics. Stem cells are capable of indefinite self-renewal; however, they still have the capability to transform into specialized cells (Martin, 1981). When stem cells transform into another cell type, the action is defined as differentiation (Odorico et al., 2001). Stem cells can undergo multi-lineage differentiation (Bunnell et al., 2008), meaning that these cells can become a specialized cell type from multiple lineages (such as bone or fat) when induced in certain culture conditions.

There are two main types of stem cells, embryonic stem cells and adult stem cells. Embryonic stem (ES) cells are cells that are present in an embryo before they differentiate into the three germ layers that form the embryo (Evans and Kaufman, 1981). Adult stem cells, the second type of stem cells, can be found in adult tissues. In an adult, stem cells are mainly for repairing and maintaining the adult tissues (Odorico et al., 2001). While ES cells can differentiate into any cell type, most adult stem cells can differentiate into a range from one specialized cell to many specialized cell types (Sell, 2005). ES cells' distinctive ability to potentially differentiate into any of the three germ layers is defined as pluripotency. The major reasons for research on stem cells are to utilize their abilities to constantly renew themselves, to be cultured over multiple

passages at an undifferentiated state, and to differentiate into specific cell types (Stocum and Zupanc, 2008).

Stem cells can be isolated and cultured for use in research. They can be isolated from the embryo or adult tissue to establish cell lines of embryonic stem cells or adult stem cells, respectively. The main incentive for conducting research on stem cells is their potential roles in reparative or regenerative medicine. The ability of the cells to differentiate into specific cell types is desirable for medicinal research. Stem cells may be used to repair injured tissues in patients with irreparable injuries or diseases (Takahashi and Yamanaka, 2006). Another suggestion is to use stem cells to better understand cellular functions such as DNA methylation or histone acetylation (Stocum and Zupanc, 2008). DNA methylation and histone acetylation are involved in embryo differentiation. Stem cells can have various uses in research because of their ability to be isolated and maintained in culture.

Embryonic Stem Cells

ES cells originate from the inner cell mass of blastocysts (Martin, 1981). As the ES cells in the inner cell mass differentiate, they are able to form the three germ layers of the embryo (endoderm, mesoderm, and ectoderm) (Evans and Kaufman, 1981). Because of this ability to differentiate into the three germ layers, ES cells are defined as being pluripotent. Another unique characteristic of ES cells is their capability of infinite self-renewal in culture (Odorico et al., 2001; Stocum and Zupanc, 2008).

ES cells were not isolated or cultured until 1981. Evans and Kaufman (1981) were able to isolate ES cells from mouse blastocysts and establish a cell line as a pluripotent tissue culture. When a blastocyst was plated, the inner cell mass from the mouse blastocyst formed egg shaped like structures inside of the growing trophoblast cells. Pluripotent cells found in the inner cell mass of the blastocyst were observed to produce ES cells (Evans and Kaufman, 1981). In the same year, ES cells were discovered to form into a wide range of cell types and formed teratomas when injected into mice (Martin, 1981). The cells were determined to contain characteristics similar to teratomacarcinoma cells. Martin et al., (1981) designated the cells derived from the inner cell mass ES cells. When cultures of the ES cells were allowed to develop into embryoid bodies, a variety of differentiated cell types were found in culture (Martin, 1981). The ability to form embryoid bodies that contain multiple differentiated cell types from the three germ layers makes the cells derived from the inner cell mass pluripotent.

Despite the observance of mouse ES cells, human ES cells were not isolated until 1998. Cells were isolated from embryos from *in vitro* fertilization (IVF) that were cultured to the blastocyst stage. The inner cell mass was revealed to produce cell lines that had similar morphology to reported ES cells in primates (Thomson et al., 1998). The lines that were established had normal karyotypes, expressed high levels of telomerase activity, expressed alkaline phosphatase, and expressed cell surface markers similar to those expressed by

nonhuman primate ES cells and human embryonal carcinoma cells (Thomson et al., 1998).

The ability of ES cells to differentiate into all three germ layers was an incredible discovery and led to a huge spark in interest in studying embryonic stem cells (Thomson et al., 1998). The inner cell mass was found to be similar to the totipotent zygote nucleus in its ability to produce a developed blastocyst and embryo (Illmensee and Hoppe, 1981). This statement has since proven to be false because ES cells cannot differentiate into the placenta. The zygote is totipotent, forming the whole embryo and placenta; however the blastocyst contains the inner cell mass and the trophectoderm. The trophectoderm will become the placenta and the inner cell mass will become the embryo. The he absence to form the extra-embryonic structures makes them pluripotent rather than totipotent. Pluripotent cells were recorded to be present in the mouse embryo until the early pre-implantation stage (Evans and Kaufman, 1981). When the inner cell mass is grown on irradiated mouse embryonic fibroblasts (MEF), ES cells can be cultured and maintained (Stocum and Zupanc, 2008).

Potential Problems with Embryonic Stem Cells

Stem cells are the driving force behind regenerative medicine. Regenerative medicine is based on using stem cells or pluripotent cells that can regenerate or repair damaged tissue. Diseases or injuries involved in dysfunction and damage of cells are potentially treatable with stem cell regenerative medicine (Odorico et al., 2001). All regenerative therapies rely on

ES cells or adult stem cells (Stocum and Zupanc, 2008). Within regenerative medicine, stem cells or artificial tissues produced from stem cells must be transplanted or implanted into the patient in order to attempt to repair the damaged tissue. An ideal source of stem cells would be one that does not produce an immune rejection response by the patient (Sun et al., 2009). While stem cells have the potential to change the scientific world with the regenerative medicine, the cells do pose some problems in practice.

There are a few obstacles that need to be considered and observed before stem cells can be used in regenerative medicine or research applications. First, stem cells have potential problems in regenerative medicine because it is difficult to isolate an optimal amount of cells to be used in therapy (Sun et al., 2009). They are also difficult to harvest, and are prone to differentiate when grown in culture (Stocum and Zupanc, 2008). A second problem with the use of stem cells in regenerative medicine is recipient rejection (immune rejection) once the stem cells are injected (Takahashi and Yamanaka, 2006). Third, ES cells pose the possibility of contamination from the mouse embryonic fibroblasts feeder layers (Sun et al., 2009). A fourth problem is the undifferentiated stem cells have been shown to produce tumors when injected into immunodeficient mice (Martin, 1981; Stocum and Zupanc, 2008). This poses many threats and hesitation to using ES cells in medicine. Last, there is a problem with isolating the ES cells from a blastocyst. In order to extract embryonic stem cells from a blastocyst, the embryo must be destroyed (Yu et al., 2007). This creates many ethical obstacles with using human ES cells.

Because of the controversy surrounding the use of ES cells, it is crucial to find other options to acquire cells that are similar to ES cells. The first option is to utilize adult stem cells, which can be isolated from adult tissue (Sun et al., 2009). A second option is reprogramming differentiated cells to become ES cell-like. Reprogramming somatic cells can produce cells that are morphologically and characteristically similar to ES cells (Takahashi and Yamanaka, 2006). This process of reprogramming cells can also be helpful to understand the regulation of cell differentiation (Stocum and Zupanc, 2008). While ES cells can be potentially devastating to use in medicine because of the cells' potential to produce immune rejection and tumors, a close alternative can be produced if adult stem cells or induced pluripotent stem cells are used.

Adult Stem Cells

Bone marrow transplants have been performed for over 40 years, but it was not until later on that bone marrow cells were discovered to be adult stem cells. Woddliff first described bone marrow isolation and characteristics of the cells in 1964. Bradley and Metcalf (1965) were able to observe colony formation when culturing mouse bone marrow cells on kidney and embryo feeder layers. While at the time the bone marrow cells were not thought to be stem cells, they were later discovered to contain hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) (Kuznetsov et al., 1997; Tropel et al., 2006). Furthermore, human MSCs from bone marrow were observed to differentiate exclusively into adipocytes, chondrocytes, or osteocytes when isolated and expanded (Pittenger et al., 1999). Pittenger et al., (1999) discovered human

MSCs were able to proliferate extensively, but still possessed the ability to differentiate into various cell types. These reports were important in paving the way to discover adult stem cells.

Adult stem cells are cells that are present in adult differentiated tissue. When animals need to regenerate certain tissues, they usually activate their adult stem cells and cause them to differentiate into the needed tissues (Stocum and Zupanc, 2008). Adult stem cells can be found in adipose tissue, cartilage, and bone marrow to name a few sources. Human adipose tissue is an abundant source of cells with an ability to provide large numbers of cells (Gimble and Guilak, 2003). Adipose stem cells have been previously isolated and characterized (Zuk et al., 2002; Gimble and Guilak, 2003; Bunnel et al., 2008). The adipose stem cells collected from humans were discovered to possess the ability to differentiate into multiple lineages making them multipotent (Bunnel et al., 2008). Later in 2009, bovine adipose stem cell isolation and characterization was described. Like adipose tissue in humans, bovine adipose stem cells were found to differentiate into chondrocytes, osteoblasts, and adipocytes (Picou, 2009).

An advantage to using adipose adult stem cells (contradicting with normal adult stem cells) is the easy isolation and large quantity of cells that can be obtained (Sun et al., 2009). This makes adult stem cells a desirable source for stem cell research because of the need for patient specific cells. Adult stem cells, such as adipose stem cells, can be isolated from people of all ages (Sun et al., 2009). The adipose stem cells are a reliable source for hundreds of million

cells from an individual (Gimble and Guilak, 2003). Adipose stem cells are developed from mesenchymal cells, and are multipotent as opposed to pluripotent because they have a limited number of specialized cells that they can become (Gimble and Guilak, 2003; Bunnell et al., 2008). Liposuction is a commonly performed surgery, and the adipose tissue is always discarded (Sun et al., 2009). Adipose stem cells can be isolated from the adipose tissue immediately after extraction, and the adipose tissue can provide enough cells for medical applications (Sun et al., 2009). The popularity of the fairly non-invasive procedure, abundance of discarded tissue, and ease of isolation of the stem cells make adipose stem cells an optimal cell choice for medical practices (Gimble and Guilak, 2003; Sun et al., 2009).

Reprogramming Somatic Cells

The main objective behind reprogramming somatic cells is to produce cells that are like ES cells. Having pluripotent characteristics means that the cells will be able to form the three germ layers of the embryo (endoderm, mesoderm, and ectoderm) (Evans and Kaufman, 1981). The term somatic cell refers to cells of the body, excluding germline cells.

Somatic cells have been examined for years for their ability to reprogram to a totipotent or pluripotent state. Somatic cells were thought to have irreversible cell nuclei that could not reprogram from their determined cell fate (Hochedlinger and Jaenisch, 2006). Differentiated cells are cells that have reached a specialized cell type, with some having reached their final cell fate.

The process that occurs when a cell becomes a more specialized cell type is called differentiation. The ability of a differentiated cell to reprogram to a totipotent or pluripotent state has mainly been observed using nuclear transfer procedures. More recently however, differentiated cells have been shown to reprogram to a pluripotent state when utilizing viral vector transduction. This method first described using a retrovirus containing four transcription factors that are involved in maintaining pluripotency in ES cells (Takahashi and Yamanaka, 2006). Takahashi and Yamanaka designated the cells with acquired pluripotent capabilities as induced pluripotent stem cells (iPS). Differentiated cells have been successfully reprogrammed by nuclear transfer (Briggs and King, 1952; Gurdon et al., 1958; Illmensee and Hoppe, 1981; Wilmut et al., 1997), direct reprogramming with viral vectors (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007; Lowry et al., 2008; Carey et al., 2009), fusion with ES cells (Cowan et al., 2005), and exposure to ES cell extracts (Hochedlinger and Jaenisch, 2006).

Reprogramming a somatic cell in amphibians was first performed by nuclear transplantation (Briggs and King, 1952). In 1952, a protocol for transferring a nucleus from a differentiated cell into an unfertilized ovum with an extracted nucleus had not been reported. The first hurdle was to create a protocol for nuclear transplantation. The protocol, reported by Briggs and King (1952), was designed to create minimal damage to the embryo while extracting and injecting the nuclei. Briggs and King (1952) wanted to test nuclei of differentiated cells to examine if the nucleus itself became differentiated as the

embryonic cells differentiated. A frog ovum was enucleated and the amphibian late blastula (embryonic) cells were used to provide the extracted nucleus. The ability of the cells to differentiate was observed by determining the cells ability to form an embryo. If complete differentiation could in fact occur, this would mean the donor cell was not irreversibly differentiated. The donor nuclei were able to produce cleaved ova and embryos, producing 52.8% cleaved ova with 31.9% ova producing blastulae out the ova that were enucleated and injected with a donor cell (Briggs and King, 1952). These results demonstrated that the transplanted nucleus of a donor cell, could in fact replace the ovum nucleus in an enucleated unfertilized ovum when used in nuclear transfer.

Briggs and King (1952) were the first to report success in nuclear transplantation. They discovered that donor nuclei from late blastocysts were able to produce embryos, however if a nucleus from an older embryo (around the gastrula stage) was used, embryos had abnormal development. John Gurdon et al., (1958) used nuclear transplantation to produce embryos in frogs (*Xenopus laevis)* from reprogrammed differentiated cells. The embryos with transplanted donor nuclei were able to produce normal tadpoles that were able to go through normal metamorphosis, become sexually mature, and have similar growth rate and sexual differentiation to the controls (Gurdon et al., 1958). As the donor cells matured to an older stage, the proportion of creating embryos from the transplanted embryos decreased. In the study, a total of 77 metamorphosed frogs were obtained from 2,594 nuclear transfers (Gurdon et al., 1958). This report was able to demonstrate that a nucleus from a differentiated cell,

transplanted into an ovum, is able to complete normal development. The differentiated cells must have the same genome as undifferentiated cells because of their ability to produce viable embryos, however the cells will perform different functions when at different stages in development. With this discovery, cells could be examined at different stages of development for their ability to reprogram.

Finally, mammalian somatic cells have been shown to have an ability to reprogram to produce a viable embryo. The first reprogrammed mammalian somatic cells were achieved in 1981 (Illmensee and Hoppe, 1981). Mouse oocytes, used in nuclear transfer, were injected with either trophectoderm or inner cell mass nuclei. The oocytes injected with inner cell mass nuclei produced 96 cleaved oocytes with 48 reaching a late pre-implantation stage (Illmensee and Hoppe, 1981). Out of 16 transferred embryos, three live mice were born from the oocytes injected with inner cell mass nuclei. This report was important because it revealed that nuclei of mammalian cells could be reversed. Next, the results were important because they are one of the first to examine the characteristics of the cells of the inner cells mass. Additionally, Wilmut et al. (1997) were the first to report a viable offspring from nuclear transfer with a differentiated adult cell from an ewe. The donor cells were adult mammalian cells from a six year-old ewe. This experiment was able to reveal that somatic cells from a mammal, other than mice, were able to reprogram (Wilmut et al. 1997). These reports establish that somatic cells are reversible regarding genetic modifications. The

differentiated cells are in fact able to reprogram to a totipotent state and produce live offspring when used in nuclear transfer.

Wilmut et al., (1997) were able to pave the road for further experiments using reprogrammed differentiated cells. With the discovery of somatic cells' ability to reprogram with nuclear transfer, the nuclei were observed to have a reversible genome able to create a viable embryo. This discovery was able to bypass the immunorejection and ethical issues concerning ES cells (Stocum and Zupanc, 2008). The differentiated nucleus of a somatic cell is able to reprogram to a totipotent state in order to de-differentiate to produce a viable offspring. The nuclei of the somatic cells have been shown to reprogram when induced by injecting them into an enucleated unfertilized oocyte.

While nuclear transfer is an acceptable procedure for producing transgenic animals or multiple clones of a superior animal, the procedure has some drawbacks. Nuclear transfer produces ethical concerns in regards to producing copies of a particular individual, which will prevent it from being accepted for use in human medicine. Another disadvantage in nuclear transfer is the low reprogramming efficiencies and low viable offspring numbers. The percentage of embryos that live to term is very low at 2-10%; this is not including the high postnatal deaths that also occur (Rideout et al., 2001). The low efficiencies and ethical concerns will keep nuclear transfer from becoming a routine clinical procedure (Odorico et al., 2001). These aspects have led to a need for a more acceptable procedure to produce pluripotent cells, such as the use of induced pluripotent stem cells.

Induced Pluripotent Stem Cells

A major focus and popular topic in human medicine today is induced pluripotent stem cells. These cells are somatic cells, not pluripotent, are reprogrammed to possess pluripotent capabilities, and have been referred to as induced pluripotent stem cells (Takahashi et al., 2007). Induced pluripotent stem (iPS) cells have quickly sparked the interest of the medical and scientific communities. The significance of iPS cells has impacted reparative or regenerative medicine, which results in customized transplantation therapy (Wernig et al., 2007). A goal of reparative medicine is to be able to repair damaged tissue, such as a spinal cord, on a patient using the patient's own cells. This technique would reduce the risk of immune rejection and would avoid the use of ES cells (Yu et al., 2007). A way to produce these patient specific ES cell-like cells is to induce the person's extracted and isolated cells to become pluripotent by viral vector transduction. Another way to utilize iPS cells is to generate iPS cells from patients with genetic diseases and investigate the disease or development new drug treatments (Park et al., 2008). While direct reprogramming (or viral vector transduction) creates cells that are like ES cells in differentiation capabilities, the mechanism of actually producing the cells is still unknown. However the process is a different reprogramming process than that involved in somatic cell nuclear transfer (Stocum and Zupanc, 2008).

The viral vector system used to create iPS cells consists of plasmids containing transcription factors that will infect 293T cells. The plasmids will assemble a virus inside of the 293T cells, and the viral particles will then lyse the

293T cells. Next, the viral particles (collected from the culture medium) can be treated on the target cells to result in integration of the viral vectors into the cells' genomes. The factors should cause endogenous expression of the cells' own genes to result in the cells having pluripotent capabilities.

The viral vectors used when inducing the somatic cells contain pluripotency transcription factors that are expressed in ES cells early in development. In order to create this system, the transcription factors must first be selected, isolated, and integrated into the plasmids and viral vectors. In order to determine the transcription factors responsible for inducing pluripotency in somatic cells, Takahashi and Yamanaka (2006) tested 24 factors. The retroviral infection was tested by the ability to select for a specific selection agent, which would result in the iPS cells becoming resistant to the agent. When all 24 factors were used in the retroviral infection on mouse embryonic fibroblasts, resistant colonies were formed. iPS cells were morphologically similar to ES cells, exhibiting a round shape, large nucleoli, and little cytoplasm (Takahashi and Yamanaka, 2006).

Next, the 24 genes were narrowed down to 10 transcription factors vital to producing induced pluripotent stem cells. When only these 10 genes were used in retroviral infection, more cell colonies were formed than when 24 genes were utilized (Takahashi and Yamanaka, 2006). This then provided a pool of 10 factors, which could be examined and narrowed down further to determine their importance in pluripotency by subtracted them one by one. No colonies were formed when Oct4, Klf4, or c-Myc was removed, but Sox2 removal resulted in a

few resistant colonies (Takahashi and Yamanaka, 2006). The only difference was found in c-Myc removal producing colonies that were not similar to ES cells. The genes essential to pluripotency were discovered to be Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006).

Morphology alone however, cannot determine a comparable similarity between iPS cells and ES cells. Therefore, quantitative RT-PCR must be performed in order to determine the presence and levels of expression of ES cell markers. Many of the ES cell marker genes were found to be expressed in higher concentrations in the pluripotent cells compared to the ES cells in the study performed by Takahashi and Yamanaka (2006). The promoters for Oct4 and Nanog were also discovered to have increased histone acetylation and decreased demethylation (Takahashi and Yamanaka, 2006). Last, iPS cells were also tested for their ability to form teratomas when injected into nude mice. The teratomas contained cells from all three germ layers, and one line of iPS cells could result in differentiation into all three germ layers after 30 passages (Takahashi and Yamanaka, 2006).

This report was a turning point, and scientifically ground breaking. The ability to produce iPS cells, and the fact that the iPS cells were indistinguishable from ES cells attracted the attention of many. Somatic cells had previously been shown to reprogram to a totipotent state using nuclear transfer (Wilmut et al., 1997), but now it was possible to reprogram somatic cells towards pluripotency without destroying embryos.

After Takahashi and Yamanaka (2006) reported the successful reprogramming of mouse cells with the four factors, the next step would be to examine human somatic cells for their ability to become pluripotent after viral infection. A retroviral transduction system was used in order to create induced pluripotent cells similar to the first experiment. The human dermal fibroblasts were introduced to a green fluorescent protein (GFP) along with the amphotropic retrovirus containing Oct4, Sox2, Klf4, and c-Myc (Takahashi et al., 2007). Expression of the green fluorescent protein would mean that the cells had reprogrammed. Despite the observations in mouse cells, less than 20% of the infected human dermal fibroblast expressed GFP (Takahashi et al., 2007). Since the reprogramming efficiencies were so low, a mouse receptor for the retrovirus was introduced into the human dermal cells and the cells were called HDF-Slc7a1 (Slc7a1 being the receptor). A mouse receptor was needed in the human dermal cells to bind the mouse Oct4 transcription factor in the amphotropic retrovirus. The human iPS cells (originally HDF-Slc7a1 cells) were similar to human ES cells in morphology, growth, and differentiation in certain culture conditions (Takahashi et al., 2007).

The iPS human cells were found to express markers observed in ES cells as high as or equivalent to the expression found in ES cells (Takahashi et al., 2007). To determine the infected cells pluripotent characteristics, the cells were allowed to form an embryoid body and then differentiate in culture. This resulted in differentiated cells from all three germ layers. Additionally, the question arose on somatic cells' reaction to the viral system. The retrovirus was also able to

result in the reprogramming of human neonate fibroblasts and primary human fibroblast-like synoviocytes to a pluripotent state (Takahashi et al., 2007).

The reports by Takahashi and Yamanaka (2006, 2007) sparked a huge interest and shift in research to focus on creating iPS cells. These reports are important to establish iPS cell characteristics and direct reprogramming protocols. The iPS cells in the experiments were altered somatic cells that were similar to ES cells in morphology, proliferation, gene expression, promoter activities, and differentiation. Scientists began working to produce pluripotent stem cells in their own labs. Mouse embryonic fibroblasts (MEF) and mouse tail-tip fibroblasts were shown to develop pluripotent characteristics when using a similar retrovirus containing Oct4, Sox2, Klf4, and c-Myc (Wernig et al., 2007). The colonies were observed to be similar to ES cell colonies by gene expression analysis using selection of colonies that expressed Oct4 and Nanog. The cells were able to form the three germ layers of an embryo (Wernig et al., 2007). Lowry et al., (2008) followed the new trend as well, and were able to create human pluripotent cells from human dermal fibroblasts. A viral system containing the four factors was used to infect the human dermal fibroblasts like the previous reports. After infection with the retrovirus containing Oct4, Sox2, Klf4, c-Myc and NANOG, colonies similar to human ES cells were observed, and normal human ES cell markers were expressed (Lowry et al., 2008).

Oct4, Sox2, Klf4, and c-Myc are referred to as the “Yamanaka four factors”, and they previously were thought to be the only factors that could produce pluripotent stem cells. While Oct4, Sox2, Klf4, and c-Myc have been

shown to be able to produce iPS cells when used in a viral system (Takahashi and Yamanaka, 2006, 2007), pluripotency can be achieved when using Oct4, Sox2, NANOG, and LIN28 (Yu et al., 2007). A lentiviral vector was used with the four factors to treat human fibroblasts. A lentivirus is a virus that can infect dividing and non-dividing cells. The experiment resulted in pluripotent cells that could differentiate into all three germ layers, and had similar morphological characteristics and a gene expression to ES cells (Yu et al., 2007). This study was important to establish the role that other transcription factors play in producing and maintaining pluripotent cells. Although Oct4 and Sox2 have been found to be essential in formation of pluripotent stem cells, LIN28 was found to be unnecessary for the initial reprogramming but it can increase reprogramming frequency when present (Yu et al., 2007). Oct4, Sox2, Klf4, and c-Myc or Oct4, Sox2, LIN28 and NANOG can induce epigenetic reprogramming to a pluripotent state by causing a change to the DNA expression rather than to the DNA sequence (Wernig et al., 2007). Park et al., (2008) collected dermal fibroblasts or bone marrow-derived mesenchymal cells from genetic disease patients for example, Parkinson disease, Huntington disease, Down syndrome, and juvenile-onset, type I diabetes mellitus to generate iPS cells using the “Yamanaka four factors” or three factors (excluding c-Myc). These cells could be used to generate cells that can be observed for the disease characteristics along with drug therapy.

Despite the major advances made with iPS cells, there is still an issue with the number of plasmids needed to construct the viral vector. Carey et al., (2009)

stated that the use of multiple proviral integrations (a single vector for each factor) could lead to insertional mutagenesis. This mutagenesis can greatly compromise the use of iPS cells in regenerative medicine. In order to decrease the number of vectors, the four factors need to be in one vector. Previously, common practice was to have one vector per factor, which would lead to multiple proviral copies, integration of oncogenes, and reactivation of silenced viral transcripts (Carey et al., 2009). To avoid these deleterious effects, a vector was constructed containing the four factors (Oct4, Sox2, Klf4, and c-Myc) that were separated by three different 2A (P2A, T2A, and E2A) peptides (Carey et al., 2009). The plasmid was used to construct viral particles, and then the particles were used to infect MEF and mouse tail tip fibroblasts. The levels of Oct4 and Sox2 in the iPS cells were similar to those of ES cells (Carey et al., 2009). Teratomas (differentiating into all three germ layers) were formed when the iPS cells were injected into immunodeficient mice. While the efficiency in this experiment was low (0.0001%), this single polycistronic vector can reprogram somatic cells and help in simplifying the infection mechanism (Carey et al., 2009).

Now that the ability to produce human and mouse pluripotent cells from fibroblasts has been reported (Takahashi and Yamanaka, 2006, 2007; Wernig et al., 2007; Yu et al., 2007; Lowry et al., 2008; Carey et al., 2009), it is important to look at other somatic cells for their ability to reprogram to a pluripotent state. Fibroblasts are the most common cell types used in reprogramming studies; however, it takes weeks to culture and establish before being able to treat them

with viral particles. Sun et al., (2009) showed a great alternative to fibroblasts with the use human adipose stem cells to produce iPS cells. Human adipose stem cells (collected by lipoaspiration) were able to reprogram two fold as fast and 20-fold more efficient when using a lentivirus containing Oct4, Sox2, Klf4, and c-Myc (Sun et al., 2009). The pluripotent cells could differentiate into cells of the three germ layers, and the iPS cells expressed pluripotency markers similar to ES cell expression (Sun et al., 2009). Importantly, human adipose stem cells can be cultured on Matrigel, eliminating the possibility MEF contamination. This report was one of the first to compare adipose stem cells to the normally used fibroblasts cells. It showed the potential of adipose stem cells in the viral vector infection procedures.

Induced pluripotent stem (iPS) cells have been produced in humans and mice, but there is little evidence of iPS cells produced in any other mammals. Finally, in 2009, iPS cells were produced in another mammal, porcine (Ezashi et al., 2009; West et al., 2009). Four lentiviral vectors containing human genes for Oct4, Sox2, Klf4, and c-Myc were used to infect porcine fetal fibroblasts, which resulted in a reprogramming efficiency of 0.1% (Ezashi et al., 2009). The colonies were found to be similar to ES cell in morphology (Ezashi et al., 2009). Also, porcine mesenchymal cells derived from bone marrow were infected with lentiviral vectors containing POU5F1, NANOG, SOX2, LIN28, KLF4, and C-MYC to produce induced pluripotent stem cells (West et al., 2009). West et al., (2009) took their experiment a step further and injected the pluripotent stem cells into pre-implantation stage embryos to produce chimeric offspring. The production of

a chimeric offspring tested the induced porcine stem cells' pluripotent capabilities. These reports lead to a hope that the success with iPS cells found in porcine cells can be discovered in other mammalian cells as well.

Some problems with iPS cells however are evident. The cells have a low reprogramming rate of less than 0.3%, and take longer than other methods to completely reprogram (Stocum and Zupanc, 2008). This makes the viral infection reprogramming a slow and inefficient process (Huangfu et al., 2008). Another major problem with iPS cells is the use of oncogenes as transcription factors in the viral system. The retrovirus-transduced oncogenes provide huge barriers to utilizing the induced cells in reparative medicine (Wernig et al., 2007). The known oncogenes are c-Myc and Klf4, and they can pose huge threats when used in the viral system (Huangfu et al., 2008). The integration of the known oncogenes into the genome of the target cells limits the use of the cells in research and clinical studies (Yu et al., 2009). In order to produce an iPS cell line to be used in therapy it could take up to two years because of the needed testing to make sure the cells would not form cancerous tumors when injected (Stocum and Zupanc, 2008). Another issue is the lack of research done on iPS cells. There has been little research conducted on transplanted iPS cells will act in the body over long periods of time, and questions such as whether the cells will lose their capability to renew themselves in response to an injury have yet to be answered (Stocum and Zupanc, 2008).

Recently, scientists have strived to correct some of the problems aforementioned to reduce some of the hesitation of using the iPS cells in

medicinal practices. iPS cells have been created without c-Myc in mouse and human fibroblasts (Nakagawa et al., 2008). This has been achieved by using transcription factors Oct4, Sox2, NANOG, and LIN28 (Yu et al., 2007). Pluripotent cells have also been generated without c-Myc in mouse embryonic fibroblasts (MEF) by using a histone deacetylase inhibitor, which improved the reprogramming ability by 50-fold as compared to the controls infected only with the three factors (Huangfu et al., 2008). While several groups have successfully produced iPS cells without c-Myc, the time it took to reprogram the cells was much longer compared to the time the procedure required when c-Myc was present (Wernig et al., 2008). Removing c-Myc from the viral vectors reduces the probability of tumor producing iPS cells. Another significant advancement was made when Huangfu et al., (2008) were able to produce induced pluripotent stem cells from primary human fibroblasts without using Klf4 or c-Myc. They utilized a histone deacetylase inhibitor to reach their results. Other attempts have been made to produce pluripotent stem cells without viral vectors. These methods use plasmids alone to transfect the target cells, however they still use integration of c-Myc and Klf4 (Okita et al., 2008). One study resulted in the derivation of human iPS cells from non-integrating episomal vectors (Yu et al., 2009). iPS cells were also created from MEF by treating the cells with recombinant proteins (Zhou et al., 2009). The recombinant proteins used were the four factors, and they were also able to create pluripotent cells when using recombinant proteins for Oct4, Sox2, and Klf4 with the use of a histone

deacetylase inhibitor. By avoiding potential integration of the known oncogenes into the target cells, the possibility of utilizing iPS cells in medicine is rising.

Chromatin Modifications of Reprogrammed Cells

Given that the generation of patient-specific stem cells can bypass the practical and ethical issues concerned with ES cell use (Carey et al., 2009), inducing somatic cells to become pluripotent has great potential in research applications. Understanding how somatic cells reprogram is a crucial first step toward improving reprogramming efficiency. It has been proposed that the low number of viable embryos produced in somatic cell nuclear transfer is a result of incomplete nuclear reprogramming (Rideout et al., 2001). Incomplete epigenetic reprogramming is considered the primary reason for developmental failure of NT embryos (Li et al., 2003). Epigenetic changes refer to heritable changes in gene function without causing a change to the DNA sequence (Kiefer, 2007). The chromatin will change but the original DNA sequence will not. Chromatin configurations that physically change the DNA are controlled by histones. Eight histones are enveloped with small fragments of DNA in an area that is called the nucleosome (Stocum and Zupanc, 2008).

Epigenetic reprogramming, discussed above, is a result of DNA methylation and histone acetylation. These modifications will result in changes to the chromatin configuration. The somatic cell nuclei must undergo epigenetic reprogramming in order to successfully reprogram to a pluripotent state. When cells are grown in vitro, it has been observed that they will show a change in

levels of DNA methylation (Allegrucci et al., 2007) and histone acetylation over time (Giraldo et al., 2007). Histone acetylation and DNA methylation need to be studied in order to better understand their role in epigenetic modifications of the developing embryos and differentiated cells. Cells can be treated with small molecules that can inhibit the chromatin-modifying enzymes to reduce the chances of having incomplete chromatin modification (Stocum and Zupanc, 2008). Manipulating the nuclear modifications with inhibitors may produce higher reprogramming efficiencies.

In gametes DNA methylation is high, but after fertilization the embryo will become demethylated (Yang et al., 2007). Methylation patterns must be erased before the embryo implants to facilitate the establishment a new methylation pattern (Rideout et al., 2001). During cleavage, DNA undergoes demethylation followed by inherited remethylation patterns between implantation and gastrulation (Ovitt and Schöler, 1998; Blelloch, et al., 2006). In an adult, mature cells have a high level of DNA methylation. In order for a somatic cell to reprogram efficiently, the DNA must be demethylated and then remethylated. The DNA is methylated by DNA methyltransferase I transferring a methyl group onto the fifth position of a cytosine on the CpG dinucleotide (Kiefer, 2007). CpG islands regulate the methylation of gene expression (Allegrucci et al., 2007). DNA methyltransferases, or DMNTs, induce silencing by interfering with the transcription factors ability to bind the DNA and by recruiting co-repressor complexes that contain histone deacetylases or histone methyltransferases (Kiefer, 2007). DNA methylation results in chromatin becoming compacted.

When DNA is methylated gene expression is repressed, resulting in the gene being silenced.

Reprogramming efficiencies of nuclear transfer embryos have been previously described as very low. This can be due to an inability of the embryos to reprogram the injected donor cell nuclei. Nuclear transfer oocytes have been observed for their ability to develop when injected with donor cells possessing low DNA methylation or high DNA methylation. It was observed that when donor cells had hypomethylated DNA, a higher number of embryos developed (Giraldo et al., 2008). An inhibitor of DNA methylransferase I can be used to treat the cells as another way to produce lower DNA methylation levels and higher reprogramming efficiencies. Blelloch et al., (2006) used an inhibitor of DNA methyltransferase I on neural stem cells to analyze whether treating the donor cells first with the inhibitor would result in higher reprogramming. The blastocysts obtained were then explanted onto MEF to observe the production of ES cell lines by the neural stem cells. The neural stem cells produced an average efficiency of 64% from the 22 neural stem cell blastocysts, which was higher than the 50% obtained from mouse fibroblasts blastocyts (Blelloch, et al., 2006). Next, donor cells were treated with a hypomorphic allele of DNA methyltransferase I prior to injecting them into the oocyte. Nuclei from fibroblasts, either control fibroblasts or hypomethylated, were used as donor cells for nuclear transfer. The hypomethylated fibroblasts had a three-fold increase in efficiency when observing ES cell derivation (Blelloch, et al., 2006). The level of

differentiation and the level of methylation of the donor cells have impacts on the efficiency of the reprogramming ability of nuclear transfer (Blelloch et al., 2006).

Zebularine, a DNA methyltransferase I inhibitor, has also been used as a treatment to maintain demethylation of cultured cells. Zebularine is a cysitidine analogue containing a pyrimidinone ring (Yoo et al., 2004). Chang et al., (2004) used zebularine to suppress methylation in human bladder cancer cells. One treatment of zebularine on bladder cancer cells resulted in a hypermethylated p16 gene (Cheng et al., 2004). A problem with this treatment is remethlyation, which can be overcome with continuous exposure. The T24 cells (bladder cancer cells) could be cultured overtime in zebularine because the chemical is stable and non-toxic. Other DNA methyltransferase inhibitors cannot be used clinically because of their instabilities and toxicities (Yoo et al., 2004). Zebularine is very stable, non-toxic, and has a half-life of 44 hours. DNA methyltransferase 1 (DNMT1) was completely depleted when using continuous treatment with zebularine (Cheng et al., 2004), allowing the tumor suppressor gene p16 to be expressed. Therefore, zebularine has great potential for cancer treatment. If p16 is expressed, it can keep the cancer cells from rapidly growing and may result in fewer cancer cell numbers. Zebularine has a potential for clinical use as a chemotherapeutic agent to reverse hypermethylation and reactivate regulatory genes in cancer cells (Yoo et al., 2004). DNA methyltransferase inhibitor, zebularine, has a potential role in cancer treatment as well as a potential role in research for inducing certain methylation patterns in somatic cells.

The second epigenetic modification that must be addressed is histone acetylation. Histone acetylation and deacetylation are important modifications involved in reprogramming and differentiating the cell nucleus. An octamer of histones (H2A, H2B, H3, and H4) is enveloped with small fragments of DNA in an area that is called the nucleosome (Stocum and Zupanc, 2008). The histones have a C-terminal tail and an N-terminal, with the N-terminal tail being subject to posttranslational modifications like acetylation (Keifer, 2007). Gametes have histones that are acetylated as a result of the chromatin becoming highly acetylated after fertilization (Rybouchkin et al., 2006). In bovine, there is reduced histone acetylation at the four-cell stage, but it is then followed by an increase in acetylation at the eight-cell stage (Yang et al., 2007). Histone acetylation is performed by adding an acetyl group to the positively charged lysine in the histone's core. This will cause the DNA to become more relaxed and able to bind the transcription activation complex in order to result in gene expression.

In order to reprogram differentiated cells, their histones must be acetylated to allow gene expression. Deacetylation in mice embryos was observed after nuclear transfer along with treatment of trychostatin A (TSA), a histone deacetylase inhibitor. Blastocyst rates as well as hatching rate were higher for the TSA treatments group when compared to the control (Rybouchkin et al., 2006). The TSA group had an increase in cell numbers, implantation rates, in blastocysts rates, and had an increase in full-term development to a fetus in the treated oocytes (Rybouchkin et al., 2006). This report reveals that histone acetylation can improve reprogramming efficiency of the donor nucleus to an ES

cell state. A similar studied performed somatic cell nuclear transfer using Cytochalasin B prevents the extrusion of the second polar body following activation and Trichostatin A on pig oocytes after activation (Beebe et al., 2009). When cytochalasin B and TSA were combined for treatment of the embryos, there was no difference in cleavage number, however a three-fold increase the number of blastocysts, and an increase in cell numbers in the blastocysts were reported (Beebe et al., 2009). The cloning efficiency of cloned calves was increased (from 2.6 to 13.4%) when a combination of TSA and 5-aza-2'-deoxycytidine (DNA methyltransferase inhibitor) treatment on donor cells and early cloned embryos was used to observe in vivo development (Wang et al., 2011). While trichostatin A has been shown to result in increased blastocysts rates and full-term development rates, there are other histone deacetylase inhibitors to consider.

Huangfu et al., (2008) tested the effect of chromatin modification inhibitors on reprogramming ability in mice cells. 5'-azacytidine (DNA methyltransferase inhibitor), Dexamethazone (synthetic glucocorticoid), and valproic acid (VPA, a histone deacetylase inhibitor) improved the rates of reprogramming by ten-fold, 2.6-fold, and >100-fold respectively (Huangfu et al, 2008). Mouse embryonic fibroblasts (MEF) were induced to be pluripotent with Oct4, Sox2, Klf4, and c-Myc. VPA was found to be the most efficient at assisting four-factor transduction, resulting in a 100-fold improvement in reprogramming efficiency when compared to the controls (Huangfu et al, 2008). Another milestone achieved in the study was that VPA treatment was able to induce pluripotency in MEF without the

oncogene, c-Myc, for a 50-fold increase in efficiency compared to 3 factor infection without VPA treatment (Huangfu et al, 2008). An important aspect to mention is that using VPA treatment on cells alone does not induce ES cell properties (Huangfu et al., 2008).

Valproic acid was used in two more studies on inducing pluripotency. Valproic acid, 2-propylpentanoic acid, is a short chain fatty acid that is used to treat epilepsy and bipolar disorder in humans (Phiel et al., 2001). The highly charged histones block binding of transcription factors to DNA. Histone acetylation neutralizes the positive charge on DNA and generates an open formation to cause re-expression of suppressed genes (Kuendgen et al., 2004). Somatic cells, specifically MEF, can be reprogrammed by direct delivery of recombinant cell-penetrating reprogramming proteins alongside treatment with valproic acid (Zhou et al., 2009). A study on primary human fibroblasts was performed to induce cells to become pluripotent with the use of Oct4 and Sox2 along with VPA treatment after infection. The iPS cells from the two-factor infection had ES cell-like characteristics, but had a lower efficiency rate than the 3-factor transduction using Oct4, Sox2, and Klf4 (Huangfu et al., 2008). Both experiments are important in verifying the ability of valproic acid to cause histone acetylation and increase reprogramming rates when induced with pluripotency factors. Valproic acid and zebularine have been shown to improve reprogramming efficiencies when utilizing somatic cell nuclear transfer or direct reprogramming. Both chemicals are stable, non-toxic, and have been used in

human medicine. These properties make them desirable to be utilized alongside viral transduction to improve reprogramming of somatic cells.

Gene Expression Associated with Reprogrammed Cells

Specific genes have been reported for their expression in ES cells and pluripotent cells. Three of these genes are Oct4, Sox2, and Nanog. In order for an embryo to develop, the cell fates must be determined at the correct time by these essential transcription factors. For development of an embryo into a viable blastocyst, critical molecular and regulated cellular events must take place (Ovitt and Schöler, 1998). These three genes are important for determining the cells that will become the inner cell mass from the cells that will become trophoblasts in the blastocyst. Oct4, Sox2, and Nanog are expressed in the inner cell mass, which has been described above as the source of ES cells (Evans and Kaufman, 1981) or pluripotent cells. Oct4 and Nanog promoters were found demethylated (genes are being expressed) in human ES cells (Takahashi et al., 2007). The same demethylation of the promoters can be found in iPS cells. However, MEF had methylated promoters (Wernig et al., 2007). The promoters for Oct4 and Nanog in induced pluripotent cells were discovered to have increased histone acetylation and decreased demethylation (Takahashi and Yamanaka, 2006). In order to test somatic cells for their ability to become pluripotent after direct reprogramming, these genes must be observed for their expression in the infected cells.

Oct4 is a transcription factor that regulates cell differentiation and is required for ES cell development. Oct4 is a member of the POU transcription

factor family, and may only be expressed by totipotent or pluripotent cells (Hattori et al., 2004). It is also known as Oct3 or POU5F1, and it is expressed in early embryo development (Ovitt and Schöler, 1998). Oct4 is down regulated in trophoblast cells; however it will still be expressed in the inner cell mass of the blastocyst. As cells differentiate, Oct4 expression will become suppressed (Ovitt and Schöler, 1998). Oct4 was found to be a "master regulator" in the ES cells because changing concentrations of Oct4 would change the cell's fate (Niwa et al., 2000). Oct4 is not expressed in trophoblasts, fibroblasts, the placenta, and other somatic cells (Hattori et al., 2004). Oct4 has been reported to be expressed when the trophoblast stem cells are treated with a DNA methyltransferase inhibitor and histone deacetylase inhibitor (Hattori et al., 2004). Alone, each inhibitor could not result in expression. However, repression of Oct4 led to differentiation into trophectoderm (Hattori et al., 2004). A noteworthy fact was the DNA methylation percentage of ES cells compared to trophoblast stem cells. The ES cells are 5% methylated (hypomethylated), and the trophoblast stem cells are 74% methylated (Hattori et al., 2004). Therefore DNA methylation is critical for regulating Oct4 expression. These findings present evidence that Oct4 expression is needed for somatic cells to reprogram to a pluripotent state because Oct4 is only expressed in totipotent and pluripotent cells. Oct 4 is crucial to determine embryo cell fates. Oct4 is required for the maintenance of pluripotency or for the prevention of differentiation of the pluripotent cells (Ovtitt and Schöler, 1998). Niwa et al., (2000) found that a less than two-fold increase in Oct4 expression levels causes differentiation of the embryo into primitive

endoderm and mesoderm, providing evidence that a specific and crucial amount of Oct4 is required for regulation of cell differentiation. DNA methylation and histone deacetylation represses Oct4 gene expression in the trophoblast cells (Hattori et al., 2004). Oct4 and Sox2 are critical core factors in determining pluripotency (Stocum and Zuplanc, 2008). All pluripotent and totipotent cells of the embryo and undifferentiated cells express Oct4 (Ovitt and Schöler, 1998).

A second gene expressed by pluripotent cells is Sox2. The Oct4 transcription factor has been shown to activate the transcription of other genes including Sox2 (Niwa et al., 2000). A member of the SOX family, Sox2, is an SRY-related gene (Sex Determining Region-Y) with an HMG (High Mobility Group) DNA-binding domain (Avilion et al., 2003). Sox2 is expressed in the inner cell mass of a blastocyst and is essential to ES cell identity. Sox2 expression has been reported in the inner cell mass, morula, blastocyst, and on into the neuroectoderm (Avilion et al., 2003). Sox2 is required to maintain the undifferentiated state. When Sox2 is absent, the cells will differentiate into trophoblast giant cells (Avilion et al., 2003). It has been reported that Sox2 regulates the multiple transcription factors, which in turn are responsible for regulating Oct4 expression (Masui et al., 2007). Sox2 expression is stimulated by Oct4 (Hattori et al., 2004), and they work together to regulate embryo development and cell differentiation. However, it has also been reported that Sox2 is necessary to regulate certain factors that affect Oct4 expression (Masui et al., 2007). When Sox2 is down regulated, Oct4 will be down regulated as well and will result in an up-regulation of Oct4 repressors (Masui et al., 2007). Oct4

and Sox2 may work together to maintain pluripotency and establish early embryonic cell fates and the first few lineages present in the embryo at the time of pre-implantation (Aviolion et al., 2003).

The last key factor in determining embryonic cell fate is Nanog. Nanog, named after the mythical Celtic land of the ever young, is a homeodomain protein that is responsible for maintaining pluripotency (Chambers et al., 2003). Nanog has a role in pluripotency of the inner cell mass and embryonic stem cells, and it is able to maintain pluripotent cells without the help of Leukemia inhibitory factor (LIF) (Mitsui et al., 2003). Nanog has been found in ES cells and embryonic carcinoma cells in mice and humans (Chambers et al., 2003). Nanog expression levels are low at the morula stage, however expression levels are high at the blastocyst stage implying that Nanog may be the crucial factor in distinguishing the pluripotent epiblasts (expressing Nanog) from the endoderm (Mitsui et al., 2003). If Nanog expression is suppressed, the ES cells will differentiate into endoderm cells (Mitsui et al., 2003). Nanog expression is regulated by epigenetic modifications, much like Oct4. Nanog and Oct4 are essential in maintaining pluripotent cells during early embryonic development (Hattori et al., 2007). All three genes, Oct4, Sox2, and Nanog, are essential to maintaining and determining pluripotent stem cells.

CHAPTER III
EXPRESSION OF OCT4, SOX2, AND NANOG IN BOVINE ADIPOSE STEM CELLS TREATED WITH VALPROIC ACID OR ZEBULARINE

Introduction

Stem cells are able to renew themselves constantly and are able to differentiate into multiple lineages (Bunnell et al., 2008). Adult stem cells are somatic cells that have similar characteristics of embryonic stem (ES) cells. They possess multipotent capabilities, which means that the cells are able to differentiate into multiple cells from the same tissue origin (Pittenger et al., 1999). Adult stem cells can be found numerous places in the adult body including bone marrow, blood, and adipose tissue. Characterization of adipose-derived stem cells in humans (Zuk et al.,2002; Gimble and Guilak, 2003; Bunnel et al., 2008) and in bovine (Williams et al., 2008; Picou, 2009) has been previously reported. Additionally, bovine adult stem (bADS) cells have been found to express pluripotency genes associated with ES cells (Coley, 2010). Adipose stem cells are multipotent, can be isolated fairly easy from people of all ages, and are able to proliferate extensively (Pittenger et al., 1999; Sun et al., 2009).

The aforementioned capabilities of adipose-derived stem (ADS) cells make them a desirable source for reprogramming experiments. The goal of reprogramming experiments is to transform somatic cells from a differentiated state to a pluripotent or totipotent state. If somatic cells contained pluripotent qualities, they could potentially differentiate into cells of the three germ layers that generate an embryo. Previously, somatic cells were thought to have irreversible cell nuclei that could not reprogram (Hochedlinger and Jaenisch,

2006); however, this statement has been discovered to be false. There are several ways to reprogram somatic cells: exposure to ES cell extracts (Hochedlinger and Jaenisch, 2006), fusion with ES cells (Cowan et al., 2005), nuclear transfer (Briggs and King, 1952; Gurdon et al., 1958; Illmensee and Hoppe, 1981; Wilmut et al., 1997), and direct reprogramming with viral transduction (Takahashi and Yamanaka, 2006, 2007; Wernig et al., 2007; Yu et al., 2007; Lowry et al., 2008; Carey et al., 2009). The first mammal produced by completely reprogramming a somatic cell utilizing nuclear transfer was Dolly (Wilmut et al., 1997). Until recently, nuclear transfer was the most popular procedure; however, success was reported reprogramming using viral transduction (Takahashi et al., 2006).

When somatic cells are reprogrammed, there are certain epigenetic changes or modifications that must occur in order to successfully reprogram the nucleus. The low number of viable embryos produced in somatic cell nuclear transfer is probably due to incomplete nuclear reprogramming (Li et al., 2003). These changes are adjustments to the gene function without any change to the DNA sequence (Kiefer, 2007). Epigenetic modifications will change the chromatin configuration and are a result of DNA methylation and histone acetylation. Somatic cells can be exposed to small molecules that may be able to reduce the chances of having incomplete chromatin modification (Stocum and Zupanc, 2008). Two epigenetic modifying factors are a DNA methyltranferase I inhibitor and a histone deacetylase inhibitor. DNA methyltransferase I inhibitor, zebularine (Zeb), has been shown to produce higher reprogramming efficiencies

when used in nuclear transfer (Blelloch et al., 2006) and in cancer cell culture (Cheng et al., 2004). The DNA methyltransferase I inhibitor would result in DNA demethylation, allowing the transcription factors to bind the DNA. This would result in gene expression. Similar results can be achieved with a histone deacetylase inhibitor. The histone deacetylase inhibitor would cause the histones to become acetylated and result in the DNA being able to bind the transcription factors that can cause gene expression. Trichostatin A and valproic acid (VPA) are a couple of histone deacetylase inhibitors. The use of trichostatin A has led to increased blastocysts rates and full-term development rates with nuclear transfer on pig oocytes (Beebe et al., 2009). VPA was able to improve reprogramming rates in induced pluripotent stem cells in mice (Huangfu et al, 2008; Zhou et al., 2009) and in humans (Huangfu et al., 2008). The epigenetic modifications can stimulate the cells to reprogram more efficiently by increasing the pluripotency-associated genes. VPA and Zeb have both been observed and shown to improve reprogramming efficiencies in somatic cell nuclear transfer (Blelloch et al., 2006; Beebe et al., 2009), cell culture (Cheng et al., 2004), and direct reprogramming (Huangfu et al, 2008; Zhou et al., 2009). Each chemical has been reported to be stable, non-toxic, and previously has been used in human medicine.

Three genes essential to maintaining and determining pluripotent cells are Oct4, Sox2, and Nanog. ES cells and pluripotent cells have high levels Oct4, Sox2, and Nanog. These three genes have been reported for their presence in adult stem cells, specifically bovine ADS cells (Coley, 2010). In order for an

embryo to develop, the cell fates must be determined at the correct time by these essential transcription factors. Oct4, Sox2, and Nanog have major roles in determining the cells that will become the inner cell mass, the source of ES cells or pluripotent cells (Evans and Kaufman, 1981). Oct4, a transcription factor, is a member of the POU transcription factor family (Hattori et al., 2004). Oct4 regulates cell differentiation and may only be expressed by totipotent or pluripotent cells. Sox2 is an SRY (Sex Determining Region-Y)-related gene with a HMG (High Mobility Group) DNA-binding domain (Avilion et al., 2003). Finally, Nanog is a homeodomain protein that is responsible for maintaining pluripotency (Chambers et al., 2003). All three genes, Oct4, Sox2, and Nanog, are essential to maintaining and determining pluripotent stem cells. These genes are important when considering epigenetic modifications. The levels of gene expression of the three genes of interest may be able to increase after treatment with the small molecule inhibitors in certain adult stem cells that previously expressed the pluripotency-associated genes.

In this study, three lines (representing three separate cows) of bovine ADS cells were isolated from the stromal fraction of adipose tissue and expanded through 3 passages. The cells were treated with VPA or Zeb to observe the changes in transcript levels of Oct4, Sox2, Nanog, and Poly A. The cells were treated for a period of 5, 7,10, or 14 days. Quantitative RT-PCR was performed on the cDNA, and the data was analyzed for statistical analysis. We hypothesized that treatment of the bovine DS cells with the modifiers would result in an increase in pluripotency-associated genes, Oct4, Sox2, and Nanog.

Materials and Methods

Stem Cell Isolation and Culture

Bovine adipose-derived stem (bADS) cells were isolated from adipose tissue collected from a local abattoir. Three adipose tissues were collected from the brisket of an adult cow and transported to the lab in Dubelcco's phosphate buffered saline (DPBS) with Ca^{2+} and Mg^{2+} + 2% penicillin/streptomycin (P/S) + 2% Fungizone (Gibco, 15290-018) on ice. The samples were washed twice and minced. The wash was aspirated off of the minced tissues, and the tissues were transferred to 150 mL Erlenmeyer flasks containing a collagenase solution of DPBS with Ca^{2+} and Mg^{2+}, One percent of bovine serum albumin (BSA), 0.25% collagenase type I (Gibco, 17100-017), 1% P/S, and 1% Fungizone. The Erlenmeyer flasks were placed in a continuous shaking incubator at 200 rpm at 37 °C for 2 hours. After incubation, the tissues and solutions were centrifuged twice at 1200 rpm for 5 minutes and shaken vigorously between centrifugations. The adipose tissues were discarded from the tube, and the liquids were transferred into a fat filter syringe system with a double filter consisting of 80 μm and 120 μm nylon filters. After a series of four centrifugations, aspirations and resuspensions, the cell pellets were finally resuspended in 1 mL of culture medium containing DMEM (high glucose) + 10% fetal bovine serum (FBS) + 1% Penicillin/Streptomycin (P/S). The resuspended cells were plated in individual 12.5 cm^2 flasks. After the cells were cultured for 24 hours, a primary wash of 2 mL of DPBS with Ca^{2+} and Mg^{2+} was used to rinse the flasks. After aspiration of the DPBS wash, 3 mL of fresh culture medium was added.

The adherent bADS cells were maintained by trypsin passage. When cells reached 90% confluency, the old medium was aspirated and a wash of DPBS (without Ca^{2+} and Mg^{2+}) was be added and aspirated. Trypsin (0.25% EDTA) was added to the flasks in order to disaggregate the cells. The cells were transferred to 15 mL tubes and centrifuged at 1200 rpm for 5 minutes to form cell pellets. The pellets were resuspended in 1 mL of culture medium and used to calculate the number of cells present by using a hemocytometer. The bADS cells were re-seeded at 7.0×10^6 in 25 cm^2 flasks.

Cell Cryopreservation

Trypsinizing and centrifuging the cells (as above) started the cryopreservation protocol for the cells. Once cells were pelleted, Calf serum (C/S) + 10% Dimethyl Sulfoxide (DMSO) (Sigma, No. D2650) was be used to resuspend the pellet. The resuspended cells were divided into cryovials and cooled at 1ºC/minute until reaching -80°C in a freezer. After 24 hours, the cells could be moved form the -80ºC to a liquid nitrogen tank. When thawing the cells, cells were allowed to sit a room temperature for 30 seconds before being placed in a 37ºC water bath.

Treatment with VPA or Zeb

Three different cell lines of bADS cells, representing three different animals, were exposed to 5 mM VPA, a DNA methyltranferase inhibitor, or 100 µM Zeb (Sigma Z4775-5MG), a histone deacetylase inhibitor. On Day one, 50,000 cells were plated in each well (18 in total) of a 24 well tissue culture dish. After 24 hours, the bADS cells were divided into a control group (receiving standard culture medium - DMEM + 10% calf serum + 1% Penicillin/Streptomycin), a group treated with VPA (culture medium + 5 mM VPA), and a group treated with Zeb (culture medium + 100 µM zebularine). The media was changed every three days. mRNA was isolated and converted to cDNA on days 5, 7, 10, or 14. The cDNA was then used in quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) to observe expression levels.

mRNA Isolation using Dynabeads® Oligo (dT) $_{25}$

bADS cells were used from culture for mRNA isolation using Dynabeads® mRNA DIRECT™ Kit (Invitrogen Dynal AS, Oslo, Norway). The medium was aspirated and trypsin was added to disaggregate the cells. The cells were moved to a 15 mL conical tube along with DMEM (high glucose) to inactivate the trypsin. The tube was centrifuged at 1200 rpm for 5 minutes to form a cell pellet. The cell pellet was resuspended in 1 mL of DPBS and transferred to a 1.5 mL microcentrifuge tube. After another centrifugation, the supernatant was removed and lysis/binding buffer was added. The cells were vortexed for 10 seconds,

stripped with a 21-gauge needle to damage the cell membrane, and centrifuged at 12000g for 15 seconds. After the cells were incubated for 10 minutes at room temperature, pre-washed dynabeads (washed with lysis/binding buffer twice) were added. The dynabeads and cells were placed in the rotator for 10 minutes at room temperature. The tube was then placed in a magnetic separator and the supernatant was removed. A series of washes was performed before eluting the RNA with 15 µL DEPC-treated water. The water and RNA were placed at 70 °C for 2 minutes. After placing the sample into the magnetic sample, the RNA was collected by removing the supernatant.

Converting mRNA to cDNA using iSCRIPT™ kit

The 15 µL of DEPC-treated water containing the mRNA was transferred to a PCR tube. 4 µL of iScript reaction mix and 1 µL of reverse transcriptase from the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were added to the tube. The PCR tube was placed into a Thermocyler for 5 minute at 25 °C, 30 minutes at 42 °C, and 5 minutes at 85 °C. After the cycler was finished, the cDNA was kept at -80 °C.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad) on a Roche LightCylcer® 480 II Real-Time PCR System (Roche Applied Science, Indianapolis, IN, USA) to test for changes in Oct4, Sox2, and Nanog using Poly A (Poly Adenylate Polymerase or PAP) as an internal reference gene. 1 µL

forward primer (10 mM), 1 µL reverse primer (10 mM), 6 µL nuclease free water, and 2 µL of cDNA (from sample) were added to 10 µL of SsoFast. The primers for Oct4, Sox2, Nanog, and Poly A (Table 3.1) were purchased from Invitrogen (CA, USA). A total of 20 µL was added to each well in a 96 well PCR plate. Each plate contained samples (replicated in doubles), positive controls/calibrators (replicated in triplicates), and negative controls (containing no templates). The reaction consisted of enzyme activation (30 seconds at 95 °C) and 40 cycles of denaturation (5 seconds at 95 °C) and annealing/extension (20 seconds at 55 °C). A final melt curve analysis with a final holding temperature of 40°C was performed to conclude the PCR.

The quantitative data was calculated using relative quantification - ΔΔCT method. The relative quantification method calculates a numerical value for the cDNA samples by comparing a ratio between expressions of two genes (for example Oct4 to PolyA). The basic relative quantification method performs analysis by using Fit points method for Cp and by using ΔΔCT Method. The ΔΔCT method calculates an n-fold difference by dividing the efficiency of the target gene ΔCTT by the efficiency of the reference gene ΔCTR (assuming an efficiency of 2). A ΔCT for the target gene was calculated by subtracting the CT value for the target gene from the CT value for the calibrator. Last, a ΔCR for the reference gene was calculated by subtracting the CT value for the sample from the CT value of the reference. A calibrator can be utilized in the PCR program to further normalize the data. The calibrator used was a combination of four lines of bADS cells that were untreated from passages 2-6, and used to give a consistent

ratio of target gene to reference gene expression for each run.

Table 3.1 Sequences and resulting amplicon lengths for primers used in qRT-PCR.

Gene	Primer Sequence	Amplicon Length
Oct4	Sense – GGTTCTCTTTGGAAAGGTGTTC Antisense - ACACTCGGACCACGTCTTTC	223
Sox2	Sense – AGGACTGAGAGAAAGAAGAAGAG Antisense – AAGAAAGAGGCAAACTGGAATC	215
Nanog	Sense – AATTCCCAGCAGCAAATCAC Antisense - CCCTTCCCTCAAATTGACAC	164
PolyA	Sense – AAGCAACTCCATCAACTACTG Antisense - ACGGACTGGTCTTCATAGC	169

Statistical Analysis

Statistical analysis was performed on the data using SAS®. Normalized ratios were log transformed prior to analysis by ANOVA to correct for lack of normality. A one-way ANOVA was completed to test the statistical significance, utilizing a P value of 0.05, among the control and treatment groups for each day. Furthermore, a pair wise comparison was conducted to test for significant differences between the VPA and Zeb treatments.

Table 3.2 F values and corresponding probabilities from single factor ANOVA for transcript levels for Oct 4, Sox2 and Nanog from cells after five days of treatment.

ANOVA	F Value	Pr > F
Oct4	20.99362	0.001955
Sox2	4.778699	0.057364
Nanog	9.794859	0.01289

Table 3.3 Log transformations of mean transcript levels for Oct4, Sox2 and Nanog in cells treated for 5,7,10 or 14 days. Values marked with asterisks (*) are different (P<.05) than controls for the corresponding gene after that duration of treatment.

Days of Exposure	Treatment	Oct4	Sox2	Nanog
5	Control	1.278	0.813	0.981
5	VPA	3.007*	2.792	2.639*
5	Zeb	2.858*	3.084	2.499*
7	Control	1.784	1.244	1.735
7	VPA	2.215	0.565	2.007
7	Zeb	2.499	3.330	2.147
10	Control	2.118	1.964	2.355
10	VPA	2.822	3.310	3.510
10	Zeb	2.228	2.013	2.456
14	Control	1.355	1.816	2.001
14	VPA	1.629	1.669	2.840
14	Zeb	1.259	1.208	1.646

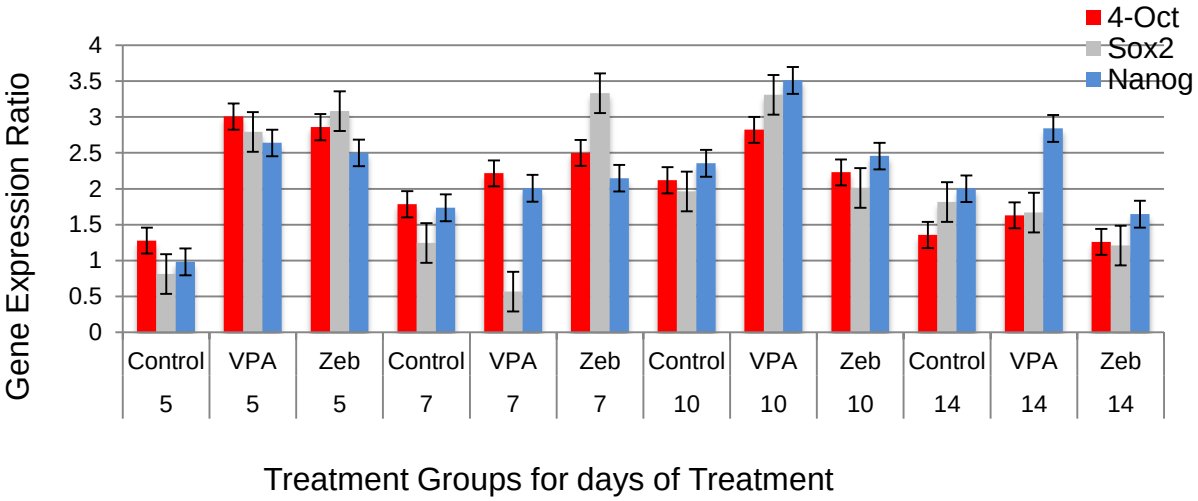

Figure 3.1. Log transformations of mean relative transcript levels (+/- SEM) for Oct4, Sox2 and Nanog in cells treated for 5,7,10 or 14 days.

Results

Significant differences in gene expression were found on Day 5 between the control and treatment groups for transcript levels of Oct4 and Nanog (Table 3.3); however, the treatments did not produce differences in gene expression levels of Oct4, Sox2, and Nanog on days 7, 10, or 14. Table 3.3 presents the ratios from this experiment with the gene expression for each treatment group over the four days.

Discussion

Adult stem cells, such as ADS cells, share unique characteristics with ES cells. These cells have been shown to possess multipotent capabilities and proliferate extensively while still possessing the ability to differentiate into various cell types (Pittenger et al., 1999). ADS cells are desirable in research due to their unique characteristics: are multipotent, can be isolated from mammals of all ages, and ease of isolation of the stem cells (Sun et al., 2009). There are several potential applications for use of ADS cells in research (Gimble and Guilak, 2003). The cells can be used to repair injured tissues in patients with irreparable injuries or diseases (Takahashi and Yamanaka, 2006). ADS cells may be advantageous when performing direct reprogramming experiments for producing pluripotent cells similar to ES cells. A third application is to use the ADS cells to better understand cellular functions such as DNA methylation or histone acetylation (Stocum and Zupanc, 2008). DNA methylation and histone

acetylation are two epigenetic modifications that must occur in the cells to successfully reprogram to a pluripotent state.

In the present experiment, bADS were treated with VPA or Zeb for a period of 5, 7, 10, or 14 days to observe the changes in Oct4, Sox2, and Nanog. The bADS cells were exposed to small molecules that may be able to reduce the chances of having incomplete epigenetic modification (Stocum and Zupanc, 2008). Zeb, a DNA methyltransferase I inhibitor, has been revealed to produce higher reprogramming efficiencies when used in nuclear transfer (Blelloch et al., 2006) and in cancer cell culture (Cheng et al., 2004). VPA, a histone deacetylase inhibitor, improved reprogramming rates in induced pluripotent stem cells in mice (Huangfu et al, 2008; Zhou et al., 2009) and in humans (Huangfu et al., 2008). Observation of Oct4, Sox2, and Nanog is essential because they play key roles in determining the pluripotent cells of the blastocyst (Evans and Kaufman, 1981), and they have been found to be present in bovine adipose-derived adult stem cells (Coley, 2010). These genes are also important when considering epigenetic modifications because the levels of gene expression may increase after treatment with the inhibitors.

When cells were treated for 5 days, Oct4 and Nanog expression levels were increased significantly ($P<0.05$) between the control and treatment groups while Sox2 was not different across treatment groups. The pair wise comparison revealed VPA and Zeb treated groups not significantly different from each other. Treatment for 7,10 or 14 days did not result in any difference in transcript levels between the treatment groups and control for any of the genes analyzed. This is

likely due to the low sample size of four cell lines. The experiment would have greater power with an increased amount of cell lines. An increase in power may reveal some statistical differences between treatments. Also, the values of gene expression ratios had large variations among each day, treatment, and cell line. For this reason, the data had to be normalized using the log transformation.

VPA and Zeb treatment for 5 days may have produced a partial reprogramming. VPA treatment led to higher Oct4, Sox2, and Nanog gene expression ratios. As shown in Table 3.3, the levels for the pluripotency-associated genes were elevated on days 5 and 10, with increased and decreased values on days 7 and 14. While this study did not find a difference in VPA treatment overall, Table 3.3 presents the ability of VPA to increase levels of gene expression for each days of exposure. VPA has been shown to cause histone acetylation and increase reprogramming rates when induced with pluripotency factors (Huangfu et al., 2008; Zhou et al., 2009). VPA has also been reported to be the most efficient at assisting four-factor transduction by producing a 100-fold improvement on reprogramming efficiencies in MEF (Huangfu et al, 2008).

The gene expression ratio levels for Oct4, Sox2, and Nanog were also increased with Zeb treatment. Notably, the values for the three genes were lower than those produced by VPA treatment (Table 3.3). Zeb treatment overall did not have a predictable pattern of influencing gene expression ratios. In previous studies, Zeb was able to suppress methylation in human bladder cells (Chang et al., 2004), and it has been reported that hypomethylation in fibroblasts

led to a three-fold increase in efficiency when observing ES cell derivation (Blelloch, et al., 2006).

In our study, bovine ADS cells treated for 5 days exhibited an increase in Oct4 and Nanog expression levels compared with the control and treatment (VPA and Zeb) groups. Sox2 was discovered to be not significant at the $P<0.05$ level ($P=0.06$). Notably, VPA and Zeb treated groups were not different from each other in their ability to impact gene expression. VPA and Zeb treatments did not produce statistical differences in gene expressions of Oct4, Sox2, and Nanog on days 7, 10,and 14. While VPA led to the highest increase of Oct4, Sox2, and Nanog, both treatments may have produced a partial reprogramming. This partial reprogramming may result in the bADS cells reaching complete pluripotency when combined with a reprogramming technique. Although further studies must be performed in order to validate this claim, this study showed that treatment of bADS with VPA or Zeb could increase the levels of pluripotency-associated genes.

CHAPTER IV

LENTIVIRAL TRANSDUCTION OF BOVINE ADIPOSE STEM CELLS TREATED WITH VALPROIC ACID OR ZEBULARINE

Introduction

Somatic cells have been examined for their ability to reprogram to a totipotent or pluripotent state. In the past, somatic cells were thought to have irreversible cell nuclei that could not be reprogrammed (Hochedlinger and Jaenisch, 2006), but that statement has been proven false. Differentiated cells have been successfully reprogrammed to a totipotent state by nuclear transfer (Briggs and King, 1952; Gurdon et al., 1958; Illmensee and Hoppe, 1981; Wilmut et al., 1997), and to a pluripotent state by direct reprogramming with viral vectors (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007; Lowry et al., 2008; Carey et al., 2009). Despite the success with reprogramming somatic cells, the reprogramming rates achieved with direct reprogramming are very low at less than 0.03% (Stocum and Zupanc, 2008). To improve the reprogramming rates, epigenetic modifications during development must be considered.

Stem cells are able to differentiate into multiple lineages and constantly renew themselves (Bunnell et al., 2008). Two forms of stem cells are adult stem cells and embryonic stem cells. Adult stem cells have similar characteristics of embryonic stem (ES) cells; particularly they possess multipotent capabilities, which enable them to differentiate into multiple cells from the same tissue origin (Pittenger et al., 1999). A unique characteristic of ES cells is their ability to

renewal themselves indefinitely in culture (Odorico et al., 2001; Stocum and Zupanc, 2008). The adult stem cells are also able to renew themselves while still maintaining the ability to differentiate into specific cell types of a certain origin (Pittenger et al., 1999). A few sources for adult stem cells in the adult body are bone marrow, blood, and adipose tissue. Characterization of adipose-derived stem cells in humans (Zuk et al.,2002; Gimble and Guilak, 2003; Bunnel et al., 2008) and in bovine (Williams et al., 2008; Picou, 2009) has been previously reported. Bovine adipose-derived stem (ADS) cells have been reported to express specific pluripotency-associated genes, Oct4, Sox2, and Nanog (Coley, 2010). The bovine ADS cells were found to differentiate into chondrocytes, osteoblasts, and adipocytes (Picou, 2009). These characteristics make bovine ADS cells an ideal source for stem cell research.

A popular focus in today's human medicine is induced pluripotent stem (iPS) cells. Somatic cells, previously not pluripotent, can be reprogrammed to become pluripotent (Takahashi et al., 2007). Reprogramming the somatic cells can be done by viral vector transduction of the cells to produce cells that have the ability to differentiate into all three germ layers. The viral vector transduction system consists of three plasmids, containing the pluripotency-associated genes that will assemble a virus inside 293T cells. Once the viruses are assembled, the viruses will lyse the 293T cells, and the viral particles can be collected along with the culture medium. The viral particles can then be used to treat target cells to cause endogenous expression of the pluripotency-associated genes, Oct4, Sox2, and Nanog, to produce iPS cells. The pluripotency-associated genes where

selected from a pool of 24 transcription factors ability to produce iPS cells from use embryonic fibroblasts. The iPS cells were described as morphologically similar to ES cells, exhibiting a round shape, large nucleoli, and little cytoplasm (Takahashi and Yamanaka, 2006). It was reported that Oct4, Sox2, Klf4, and c-Myc were essential to the production of iPS cells, and are referred to as the "Yamanaka four factors" (Takahashi and Yamanaka, 2006). Somatic cells may be observed to have similar morphology to ES cells once transformed into iPS cells; however, morphology alone cannot determine pluripotency. Quantitative RT-PCR is another means to test the cells ability to become pluripotent by examining the presence and levels of expression of ES cell markers. iPS cells have been produced using a transduction system containing Oct4, Sox2, Klf4, and c-Myc in human dermal fibroblasts (Takahashi et al., 2007; Lowry et al., 2008), mouse embryonic fibroblasts (Takahashi and Yamanaka, 2006; Wernig et al., 2007), and mouse tail-tip fibroblasts (Wernig et al., 2007).

The transduction system consisted of a plasmid for each transcription factor, Oct4, Sox2, Klf4, and c-Myc. These four plasmids provide a problem with multiple proviral integrations, which can lead to insertional mutagenesis (Carey et al., 2009). In order to avoid insertional mutagenesis, a single plasmid was constructed to contain all four factors (Oct4, Sox2, Klf4, and c-Myc) that were separated by three 2A peptides (Carey et al, 2009). Fibroblasts are the most common cell type used in reprogramming experiments with viral vector transduction. An alternative to using fibroblasts is the use of adipose stem cells (Sun et al., 2009). Human adipose stem cells were able to reprogram when

transduced with a lentivirus containing Oct4, Sox2, Klf4, and c-Myc (Takahashi et al., 2007; Lowry et al., 2008; Sun et al., 2009). Somatic cells in mice (Takahashi and Yamanaka, 2006; Wernig et al., 2007; Carey et al, 2009), humans (Takahashi et al., 2007; Lowry et al., 2008; Sun et al., 2009), and pigs (Ezashi et al., 2009; West et al., 2009) have been reported to possess pluripotent capabilities when induced using a transduction system.

While iPS cells have many advantages, they also have a few negative aspects. The viral transduction method is a slow and inefficient process because reprogramming somatic cells results in a low reprogramming rate (Huangfu et al., 2008). A second barrier with the viral system is limitations on utilization of the iPS cells in reparative medicine because of the integration of known oncogenes, c-Myc and Klf4 (Wernig et al., 2007; Huangfu et al., 2008). There has been very little research conducted on iPS cells and the medizmal affects they will have overtime. In order to correct these problems, iPS cells were produced using a histone deacetylase inhibitor (Huangfu et al., 2008; Zhou et al., 2009). The use of the histone deacetylase inhibitor allowed for c-Myc to be removed from the vector to decrease the possibility of integrating this known oncogene and enhance the chances of using iPS cells in reparative studies.

The histone deacetylase inhibitor has another role in nuclear reprogramming. When somatic cells are triggered to reprogram, there are certain epigenetic changes that must occur in order to successfully reprogram the nucleus to a less differentiated state. These epigenetic modifications change the chromatin configuration without changing the DNA sequence. It has been

shown that somatic cells can be exposed to small epigenetic modifying factors in order to facilitate these epigenetic modifications required for reprogramming towards pluripotency. Two of these epigenetic modifying factors are zebularine (Zeb), a DNA methyltranferase inhibitor, and valproic acid (VPA), a histone deacetylase inhibitor. Valproic acid and zebularine have been shown to improve reprogramming efficiencies when utilizing somatic cell nuclear transfer or direct reprogramming (Cheng et al., 2004; Huangfu et al., 2008; Zhou et al., 2009). Both chemicals are stable, non-toxic, and have been used in human medicine. These properties make them desirable to be utilized alongside viral transduction to improve reprogramming of somatic cells. By enhancing Oc4, Nanog and Sox2 expression with these epigenetic modifiers, somatic cells may be reprogrammed more effectively.

As previously reported in Chapter III, bovine ADS cell exposure to VPA and Zeb resulted in significant differences in transcript levels of Oct4 and Nanog between treatment and control groups. VPA and Zeb treatment increased the levels of the pluripotency-associated genes. For this reason, a partial reprogramming may have taken place, and complete pluripotency may be achieved when combined with viral transduction.

In this experiment, three bADS cell lines (from three separate cows) at passage 3 were treated with VPA or Zeb for 5 days before being exposed to a lentivirus containing Oct4, Sox2, Klf4, and c-Myc for 24 hours. The cells were observed for changes in cell morphology and transcript levels of Oct4, Sox2, or Nanog.

Materials and Methods

Stem Cell Isolation and Culture

Bovine adipose-derived stem (bADS) cells were isolated from adipose tissue collected from a local abattoir. The adipose tissues were taken from the brisket of an adult cow and transported to the lab in Dubelcco's phosphate buffered saline (DPBS) with Ca^{2+} and Mg^{2+} + 2% penicillin/streptomycin (P/S) + 2% Fungizone (Gibco, 15290-018) on ice. The adipose tissue samples were washed twice and minced. The wash was aspirated off of the minced tissues, and the tissues were transferred to 150 mL Erlenmeyer flasks containing a collagenase solution of DPBS with Ca^{2+} and Mg^{2+}, One percent of bovine serum albumin (BSA), 0.25% collagenase type I (Gibco, 17100-017), 1% P/S, and 1% Fungizone. The Erlenmeyer flasks were placed in a continuous shaking incubator at 200 rpm at 37 °C for 2 hours. After incubation, the tissues and solutions were centrifuged twice at 1200 rpm for 5 minutes and shaken vigorously between centrifugations. The adipose tissue samples were decanted, and the supernatants were transferred into a fat filter syringe system with a double filter consisting of 80 µm and 120 µm nylon filters. After a series of four centrifugations, aspirations and resuspensions, the cell pellets were resuspended in 1 mL of culture medium containing DMEM (high glucose) + 10% fetal bovine serum (FBS) + 1% P/S. The resuspended cells were plated in individual 12.5 cm^2 flasks. After the cells were cultured for 24 hours, a primary wash of 2 mL of DPBS with Ca^{2+} and Mg^{2+} was used to rinse the flasks. After aspiration of the DPBS wash, 3 mL of fresh culture medium was added.

The adherent bADS cells were maintained by trypsin passage. When cells reached 90% confluency, the old medium was aspirated and cells washed with DPBS (without Ca^{2+} and Mg^{2+}). Trypsin (0.25% EDTA) was added to the flasks in order to disaggregate cells. The recovered cells were transferred to 15 mL tubes and centrifuged at 1200 rpm for 5 minutes to form cell pellets. The pellets were resuspended in 1 mL of culture medium and used to calculate the number of cells present using a hemocytometer. The bADS cells were re-seeded at 7.0×10^6 in 25 cm^2 flasks in standard culture medium.

Cell Cryopreservation

Following trypsinization and centrifugation, calf serum (C/S) + 10% Dimethyl Sulfoxide (DMSO) (Sigma, No. D2650) was be used to resuspend the pellet. The resuspended cells were divided into cryovials and cooled at 1ºC/minute until finally reaching -80°C. After 24 hours, the cells were transferred form -80ºC to a liquid nitrogen tank. For thawing, cells were allowed to incubate at room temperature for 30 seconds before being placed in a 37ºC water bath.

Treatment with VPA or Zeb

Three different cell lines of bADS cells, representing three different animals, were exposed to 5 mM VPA, a DNA methyltranferase inhibitor, or 100 µM Zeb (Sigma Z4775-5MG), a histone deacetylase inhibitor. On Day 1, 50,000 cells were plated in each well (18 in total) of a 24 well tissue culture dish. After 24 hours, the bADS cells were divided into six treatment groups. The six treatment groups were a control group (receiving standard culture medium -

DMEM + 10% calf serum + 1% Penicillin/Streptomycin), a VPA treated group (culture medium + 5 mM VPA), a VPA and lentivirus treated group, a Zeb treated group (culture medium + 100 μM zebularine), a Zeb and lentivirus treated group, and a group treated with only with the lentivirus.

Producing the Lentiviral Particles

First, 700,000 293T (HEK) cells were plated in each 60mm dish in 5 mL of medium (High-glucose DMEM with 10% FBS). The next day, 2.5 μL of OSKM, 3 μL of PAX2, 5.2 μL of MD2m and 9.3 μL of OPTI-MEM were combined. Combining 6 μL Fugene with 74 μL OPTI-MEM made the master mix of Fugene 6 transfection reagent. Then the mixture of plasmids and Fugene was incubated for 5 min at room temperature. 80 μL of Fugene master mix was added to a polypropylene tube, and incubated at room temperature for 20 min. The 293T cells need to be at 50-80% confluency. Add the DNA:FUGENE® mix drop wise to the cells. Then the 293T cells were incubated at 37°C, 5% CO_2 for 12-15 hours. The next morning, the medium will be changed to remove the transfection reagent and replaced with 5mL of DMEM, 10% FBS, and 1% penicillin/streptomycin. Over the next two days, medium harvested will be collected and stored in a polpropylene storage tube at 4°C. The solution was centrifuged at 1,250 rpm for 5 minutes to pellet any 293T cells that may have been collected.

Infecting Target Cells with Lentiviral Particles

Target cells had their medium changed to fresh culture medium after 5 days of treatment, and lentiviral particles were added. For each well to be infected in the 24 well dish, 57μL of the viral particles were added. Cells were incubated for 24 hours at 37°C, 5% CO2 before changing to fresh culture medium. After 3-4 days from initial infection, the cells were moved to a BD Matrigel™ (hESC-quaified Matrix) coated dish along with embryonic stem cell medium. The ES cell medium contained High-glucose Knockout DMEM, 15% ES cell-qualified fetal bovine serum (FBS), 2mM GlutaMax-I, 1 mM non-essential amino acids, 1000 U/mL Recombinant Mouse Leukemia Inhibitory Factor (LIF), 1μM β-Mercaotoethanol, and 1% Penicillin/Streptomycin. The media was changed every two days. Cells were passaged one to two times when colonies were observed. Messenger RNA was isolated after establishing colonies after passaging and converted to cDNA. The cDNA was then used in quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) to observe expression levels.

mRNA Isolation using Dynabeads® Oligo (dT) $_{25}$

Cultured bADS cells were utilized for mRNA isolation using a Dynabeads® mRNA DIRECT™ Kit (Invitrogen Dynal AS, Oslo, Norway). The medium was aspirated and trypsin was added to disaggregate the cells. The cells were moved to a 15 mL conical tube in DMEM (high glucose) to inactivate the trypsin. The tube was centrifuged at 1200 rpm for 5 minutes, then the cell pellet was

resuspended in 1 mL of DPBS and transferred to a 1.5 mL microcentrifuge tube. After another centrifugation, the supernatant was removed and lysis/binding buffer was added. The cells were vortexed for 10 seconds, stripped with a 21-gauge needle, and centrifuged at 12000g for 15 seconds. After the cells were incubated for 10 minutes at room temperature, pre-washed dynabeads (washed with lysis/binding buffer twice) were added. The dynabeads and cells were placed in the rotator for 10 minutes at room temperature. The tube was then placed in a magnetic separator and the supernatant was removed. A series of washes was performed before eluting the RNA with 15 µL DEPC-treated water. The water and RNA were placed at 70 °C for 2 minutes. After placing the sample into the magnetic sample, the RNA was collected by removing the supernatant.

Converting mRNA to cDNA using iSCRIPT™ kit

The 15 µL of DEPC-treated water containing the mRNA was transferred to a PCR tube. Then, 4 µL of iScript reaction mix and 1 µL of reverse transcriptase from the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were added to the tube. The PCR tube was placed into a Thermocyler for 5 minutes at 25 °C, 30 minutes at 42 °C, and 5 minutes at 85 °C. After the cycler was finished, the cDNA was kept at -80 °C.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad) on a Roche LightCylcer® 480 II Real-Time PCR System (Roche Applied Science,

Indianapolis, IN, USA) to test for changes in Oct4, Sox2, and Nanog using Poly A (polyadenylate polymerase or PAP) as an internal reference gene. Next, 1 µL forward primer (10 mM), 1 µL reverse primer (10 mM), 6 µL nuclease free water, and 2 µL of cDNA (from sample) were added to 10 µL of SsoFast. The primers for Oct4, Sox2, Nanog, and Poly A (Table 3.1) were purchased from Invitrogen (CA, USA). A total of 20 µL was added to each well in a 96 well PCR plate. Each plate contained samples (replicated in doubles), positive controls/calibrators (replicated in triplicates), and negative controls (containing no templates). The reaction consisted of enzyme activation (30 seconds at 95 °C) and 40 cycles of denaturation (5 seconds at 95 °C) and annealing/extension (20 seconds at 55 °C). A final melt curve analysis with a final holding temperature of 40°C was performed to conclude the PCR.

The quantitative data was calculated using relative quantification - ΔΔCT method. The relative quantification method calculates a numerical value for the cDNA samples by comparing a ratio between expressions of two genes (for example Oct4 to PolyA). The basic relative quantification method performs analysis by using Fit points method for Cp and by using ΔΔCT Method. The ΔΔCT method calculates an n-fold difference by dividing the efficiency of the target gene ΔCTT by the efficiency of the reference gene ΔCTR (assuming an efficiency of 2). A ΔCT for the target gene was calculated by subtracting the CT value for the target gene from the CT value for the calibrator. Last, a ΔCR for the reference gene was calculated by subtracting the CT value for the sample from the CT value of the reference. A calibrator was utilized in the PCR program to

further normalize the data. The calibrator used was a combination of four lines of bADS cells that were untreated from passages 2-6, and used to give a consistent ratio of target gene to reference gene expression for each run.

Table 4.1 Sequences and resulting amplicon lengths for primers used in qRT-PCR.

Gene	Primer Sequence	Amplicon Length
Oct4	Sense – GGTTCTCTTTGGAAAGGTGTTC Antisense - ACACTCGGACCACGTCTTTC	223
Sox2	Sense – AGGACTGAGAGAAAGAAGAAGAG Antisense – AAGAAAGAGGCAAACTGGAATC	215
Nanog	Sense – AATTCCCAGCAGCAAATCAC Antisense - CCCTTCCCTCAAATTGACAC	164
PolyA	Sense – AAGCAACTCCATCAACTACTG Antisense - ACGGACTGGTCTTCATAGC	169

Statistical Analysis

Statistical analysis was completed using SAS®. The statistical significance of the experiment was set at $P \leq 0.05$. A type 3 ANOVA was performed to test for significant differences among the 6 treatments for each pluripotency-associated gene, Oct4, Sox2, and Nanog. n-fold difference = Efficiency Target Gene$^{\Delta CTT}$/ Efficiency Reference Gene$^{\Delta CTR}$

Table 4.2 P values for mean relative transcript levels of Oct4, Sox2 and Nanog compared to controls in cells treated with zebularine for five days then cultured for 14-16 days on Matrigel.

Gene	P value
Oct4	0.0160
Sox2	0.0221
Nanog	0.0065

Table 4.3 Mean relative transcript levels for Oct4, Sox2 and Nanog in control treatment and cells treated with VPA or zebularine and/or infected with lentivirus particles. Values marked with an asterisk (*) are different (P< .05) from the control treatment for the corresponding gene.

Treatment	Oct4	Sox2	Nanog
Control	65.32033333	84.81746667	47.6419
VPA only	122.4062	4.95E+01	38.87443333
Zeb only	284.3566667*	182.5593333*	116.4466667*
VPA+Lentivirus	114.1696667	31.7363	31.65406667
Zeb+ Lentivirus	157.622	81.50066667	72.28906667
Lentivirus only	100.4565333	99.23946667	67.82446667

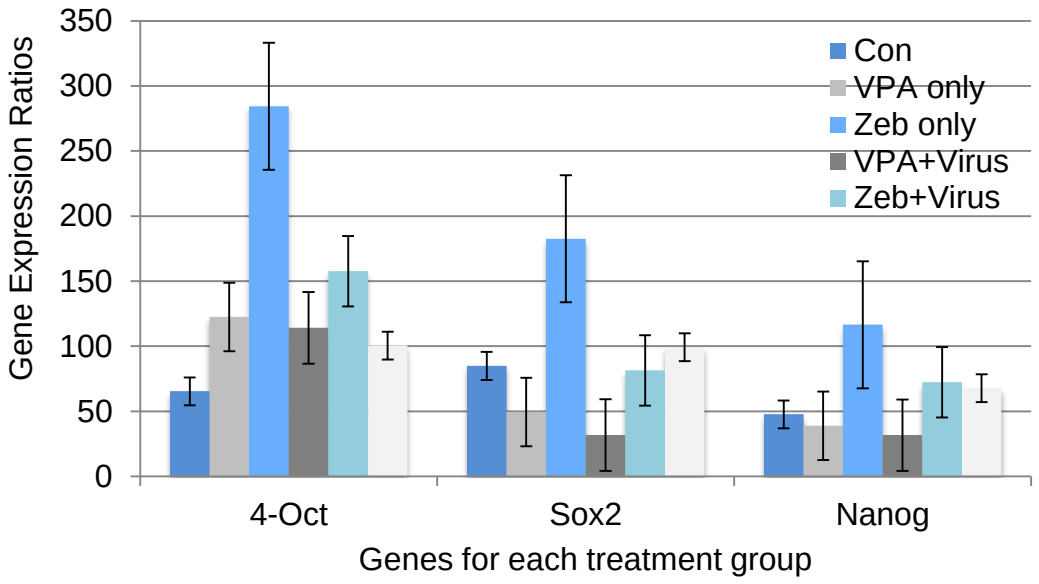

Figure 4.1 Mean relative transcript levels (+/- SEM) for Oct4, Sox2 and Nanog in control treatment and cells treated with VPA or zebularine and/or infected with lentivirus particles.

Results

There were differences in expression of Oct4, Sox2, and Nanog across treatments of the cells (Table 4.2). Zebularine treatment along with the viral infection resulted in an increase from the controls in Nanog (P=0.0592). Higher levels of pluripotency-associated genes were achieved when using epigenetic modifiers (Table 4.3); however, the gene expressions were highest with the use of epigenetic modifiers rather than epigenetic modifiers with lentivirus. Use of the lentivirus only resulted in higher expression ratio values when compared to the control.

Discussion

Adipose-derived stem (ADS) cells are a desirable source of cells for stem cell research because of their multipotent capabilities and ease of isolation (Bunnel et al., 2008; Sun et al., 2009). They are a reliable source of large quantities of cells that can be harvested from an individual (Gimble and Guilak, 2003). Reprogramming experiments aim to produce cells that are less differentiated, can utilize ADS cells with hopes to improve reprogramming efficiencies and avoid patient immune rejections. Reprogramming studies have been performed on differentiated cells in nuclear transfer (Briggs and King, 1952; Gurdon et al., 1958; Illmensee and Hoppe, 1981; Wilmut et al., 1997), direct reprogramming with viral vectors (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007; Lowry et al., 2008; Carey et al., 2009), fusion with ES cells (Cowan et al., 2005), and exposure to ES cell extracts (Hochedlinger and Jaenisch, 2006).

In this experiment, the reprogramming method of choice for the bovine ADS cells was viral vectors. Cells treated for 5 days with epigenetic modifiers were chosen for optimal expression of pluripotency-associated genes, Oct4, Sox2, and Nanog (see Chapter 3). The ability of the lentivirus to reprogram the cells was observed following treatment of the ADS cells with epigenetic modifiers (VPA or Zeb) or without modifiers. The lentiviral vector contained transcription factors for Oct4, Sox2, Klf4, and c-Myc. These factors are known as the "Yamanaka four factors" based on their ability to produce iPS able to self renew and differentiate into the three germ layers for 30 passages (Takahashi and Yamanaka, 2006). The lentiviral vector utilized in this experiment was composed of Addgene's OSKM, PAX2, and MD2m.

Gene expression was variable among cell lines and treatments in this experiment. When looking strictly at numerical data, zebularine treatment and treatment with Zeb and lentiviral particles produced the highest increases in pluripotency-associated genes. Oct4, Sox2, and Nanog were increased when epigenetic modifiers and a combination of the epigenetic modifiers along with the lentivirus was used.

Statistical differences for expression of the pluripotency-associated genes were found for cells treated with Zeb. Zebularine treatment along with the viral infection resulted in a significant increase in Nanog ($P=0.0592$). Zeb was able to deplete DNA methyltransferase 1 with continuous treatment (Cheng et al., 2004), and this ability to deplete DNA methyl transferase could be the reason in our study why Zeb led to significant increases of gene expression. While Zeb was

the only modifier to produce significant changes in this experiment, it has been reported that VPA was the most efficient at assisting four-factor transduction, resulting in a 100-fold improvement in reprogramming efficiency when compared to the controls (Huangfu et al, 2008). A reason for the lack of a difference in gene expression when using VPA alone is consistent with the finding that using VPA treatment alone on cells does not induce ES cell properties (Huangfu et al., 2008).

It was hypothesized that viral transduction in combination with epigenetic modifiers would produce higher expression levels of the genes of interest, but that was not the case in this experiment (Table 4.3). Although viral transduction or treatment of the bovine ADS cells with epigenetic modifiers may produce partial reprogramming, further investigation is needed to determine if complete pluripotency was achieved. More cell lines should be utilized to increase the power of the experiment. Induced cells also need to be examined for their ability to produce teratomas in nude mice (Takahashi and Yamanaka, 2006). While this study illustrated the ability of epigenetic modifiers and lentiviral transduction to increase pluripotency-associated genes, this is only preliminary data in the production of iPS cells able to be utilized in research for SCNT or clinical research with regenerative medicine.

CHAPTER V
SUMMARY AND CONCLUSIONS

Somatic cells were originally believed to possess irreversible cell nuclei that could not reprogram from their determined cell type (Hochedlinger and Jaenisch, 2006). However, this has was proven false with nuclear transfer (Wilmut et al., 1997) and direct reprogramming with viral vectors (Takahashi and Yamanaka, 2006). Differentiated cells can be reprogrammed to a pluripotent state, where they have the ability to differentiate into all three germ layers of an embryo. These cells can then be directed to differentiate into the desired cell type when placed in certain culture conditions. These methods of producing pluripotent cells are the driving force behind regenerative medicine because it could produce patient specific cells that can regenerate or repair the patients damaged or malfunctioning tissue. Although there is high potential with reprogramming somatic cells with viral transduction, the reprogramming efficiencies are very low with a reprogramming rate of less than 0.3% (Stocum and Zupanc, 2008). In order to increase the reprogramming rate and help decrease the need for known oncogenes (Klf4 and c-Myc) in the viral vector, cells have been treated with epigenetic modifiers. Epigenetic changes refer to heritable changes in gene function without causing a change to the DNA sequence (Kiefer, 2007). Treating the cells with epigenetic modifiers can inhibit the chromatin-modifying enzymes to reduce the chances of having incomplete chromatin modification (Stocum and Zupanc, 2008).

Bovine adipose-derived stem (ADS) cells were treated with one of two epigenetic modifiers, valproic acid or zebularine, for 5, 7, 10, or 14 days to observe the changes in pluripotency-associated genes (Oct4, Sox2, and Nanog). This first experiment produced a significant difference in cells treated for 5 days with either epigenetic modifier for Oct4 and Nanog. The treatments were found to not be significant from each other. This partial reprogramming may result in the bADS cells reaching complete pluripotency when combined with a reprogramming technique. It has been reported previously that VPA can cause histone acetylation and increase reprogramming rates when induced with pluripotency factors (Huangfu et al., 2008; Zhou et al., 2009). VPA has also been reported to be the most efficient at assisting four-factor transduction by producing a 100-fold improvement on reprogramming efficiencies in MEF (Huangfu et al, 2008). Furthermore, Zeb was observed to deplete DNA methyltransferase 1 with continuous treatment (Cheng et al., 2004).

The second experiment combined the treatment of bovine ADS with the epigenetic modifiers from experiment 1 followed by transduction of the cells with a lentivirus. Statistical differences for expression of the pluripotency-associated genes were found for cells treated with Zeb. Zebularine treatment along with the viral infection resulted in a significant increase in only Nanog (P value of 0.0592). As seen in the previous paragraph, VPA and Zeb should be able to cause the desired epigenetic changes; however, the changes in pluripotency-associated genes were variable in these experiments.

Some improvements can be made in future experiments. More cell lines should be utilized in to increase the power of the experiments. This would hopefully produce more uniform and consistent data. Induced cells also need to be examined for their ability to produce teratomas in nude mice (Takahashi and Yamanaka, 2006). While epigenetic modifiers and lentiviral transduction have been shown to increase pluripotency-associated genes, there is still a lot of improvement and some changes that can be made to these experiments.

REFERENCES

Allegrucci, C., Y. Wu, A. Thurston, C.N. Denning, H. Priddle, C.L. Mummery, D.W. Andrews, M. Stojkovic, N. Smith, T. Parkin, M. E. Jones, G. Warren, L. Yu, R.M. Brena, C. Plass, L.E. Young. Restriction landmark genome scanning identifies culture-induced DNA methylation instability in the human embryonic stem cell epigenome. Oxford University Press. 2007. Print.

Avilion, A.A., S.K. Nicolis, L.H. Pevny, L. Perez, N. Vivian, and R. Lovell-Badge. 2003. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes and Development. 17: 126-140.

Beebe, L.F.S., S.J. McIlfatrick, and M.B. Nottle. 2009. Cytochalasin B and trichostatin A treatment postactivation improves in vitro development of porcine somatic cell nuclear transfer embryos. Cloning and Stem Cells 11: 477-482.

Blelloch, R., Z. Wang, A. Meissner, S. Pollard, A. Smith, R. Jaenisch. 2006. Reprogramming efficiency following somatic cell nuclear transfer is influenced by the differentiation and methylation state of the donor nucleus. Stem Cells 24: 2007-2013.

Bradley T. R., and D. Metcalf. The growth of mouse bone marrow cells in vitro. Aust. J. Exp. Biol. Med. Sci. 44:287-300, 1966.

Briggs, R. and King, T.J. 1952. Transplantation of living nuclei from blastula cells into enucleated frog eggs. Proc. Natl. Acad. Sci. USA. 38:455-463.

Bunnell, B.A., M. Flaat, C. Gagliardi, B. Patel, and C. Ripoli. 2008. Adipose-derived stem cells: Isolation, expansion and differentiation. Methods 45: 115-120.

Carey, B.W., S. Markoulaki, J. Hanna, K. Saha, Q. Gao, M. Mitalipova, and R. Jaenisch. 2009. Reprogramming of murine and human somatic cells using a single polycistronic vector. Proc. Natl. Acad. Sci. USA. 106: 157-162.

Chambers, I., D. Colby, M. Robertson, J. Nichols, S. Lee, S. Tweedie and A. Smith. 2003. Functional expression cloning of nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113: 643-655.

Cheng, J.C., D.J. Weisenberger, F.A. Gonzales, G. Liang, G. Xu, Y. Hu, V.E. Marquex, and P.A. Jones. 2004. Continuous zebularine treatment effectively sustains demethylation in human bladder cancer cells. Mol. and Cell Biol. 24: 1270-1278.

Cowan, C. A., J. Atienza, D. A. Melton and K. Eggan. 2005. Nuclear Reprogramming of Somatic Cells after Fusion with Human Embryonic Stem Cells. Science 309: 1369-1373.

Evans, M. J. and M. H. Kaufman. 1981. Establishment in Culture of Pluripotential Cells from Mouse Embryos. Nature 292: 154-156.

Ezashi, T., B.P.V.L. Telugu, A.P. Alexenko, S. Sachdev, S. Sinha, and R.M. Roberts. 2009. Derivation of induced pluripotent stem cells from pig somatic cells. Proc. Natl. Acad. Sci. USA. 106: 10993-10998.

Gimble, J. M. and F. Guilak. 2003. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. Cytotherapy 5: 362-369.

Giraldo, A., Lynn, J.W., Purpera, M.N., Godke, R.A. and Bondioli, K.R. 2007. DNA methylation and histone acetylation patterns in culture bovine fibroblasts for nuclear transfer. Mol. Reprod. Dev. 74:1514-1524.

Giraldo, A.M., D.A. Hylan, C.B. Ballard, M.N. Purpera, T.D. Vaught, J.W. Lynn, R.A. Godke, and K.R. Bondioli. 2008. Effect of epigenetic modifications of donor somatic cells on the subsequent chromatin remodeling of cloned bovine embryos. Biol. of Reprod. 78: 832-840.

Gurdon JB,T.R. Elsdale,and M. Fischberg 1958. Sexually mature individuals of Xenopus laevis from the transplantation of single somatic nuclei. Nature 182:64–65.

Hattori, N., K. Nishino, Y. Ko, N. Hattori, J. Ohgane, S. Tanaka, and K. Shiota. 2004. Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. J. of Biol. Chem. 279: 17063-17069.

Hochedlinger, K. and R. Jaenisch. 2006. Nuclear Reprogramming and Pluripotency. Nature 441: 1061-1067.

Huangfu, D., R. Maehr, W. Guo, A. Eijkelenboom, M. Snitow, A.E. Chen, and D.A. Melton. 2008. Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. Nature Biotechnology 26: 795-798.

Illmensee, K., and P.C. Hoppe. 1981. Nuclear transplantation in mus musculus: Developmental potential of nuclei from preimplantation embryos. Cell 23: 9-18.

Kiefer, J.C. 2007. Epigenetics in development. Developmental Dynamics. 236: 1144-1156.

Knendgen, A., C. Strupp, M. Aivado, A. Bernhardt, B. Hildebrandt, R. Haas, U. Germing, and N. Gattermann. 2004. Treatment of myelodysplastic syndromes with valproic acid alone or in combination with all-trans retinoic acid. Blood 104: 1266-1269.

Kuznetsov, S.A., A.J. Friedenstein and P.G. Robey, 1997. Factors required for bone marrow stromal fibroblast colony formation in vitro. Br. J. Haematol. 97: 561-570.

Li, X., Z. Li, A. Jouneau, Q. Zhou and J.-P. Renard. 2003. Nuclear Transfer: Progress and Quandaries. Reprod. Biol. Endocrinol. 1: 84-96.

Lowry, W.E., L. Richter, R. Yachechko, A.D. Pyle, J. Tchieu, R. Sridharan, A.T. Clark, and K. Plath. 2008. Generation of human induced pluripotent stem cells from dermal fibroblasts. Proc. Natl. Acad. Sci. USA 8: 2883-2888.

Martin, G. R. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. USA 78: 7634-7638.

Masui, S., Y. Nakatake, Y. Toyooka, D. Shimosato, R. Yagi, K. Takahashi, H. Okochi. A, Okuda, R. Matoba, A.A. Sharov, M.S.H. Ko, and H. Niwa. 2007. Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. Nature Cell Biol. 9: 625-635.

Mitsui, K., Y. Tokuzawa, H. Itoh, K. Segawa, M. Murakami, K. Takahashi, M. Maruyama, M. Maeda and S. Yamanaka. 2003. The homeoprotein nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113: 631-642.

Niwa, H., J. Miyazaki, and A.G. Smith. 2000. Quantitative expression of Oct-3/4 defines differentiation dedifferentiation or self-renewal of ES cells. Nature Genetics 24: 372-376.

Odorico, J. S., D. S. Kaufman and J. A. Thomson. 2001. Multilineage Differentiation from Human Embryonic Stem Cell Lines. Stem Cells 19: 193-204.

Okita, K., T. Ichisaka, and S. Yamanaka. 2007. Generation of germline-compotent induced pluripotent stem cells. Nature 448: 313-318.

Ovitt, C. E. and H. R. Schöler. 1998. The Molecular Biology of Oct-4 in the Early Mouse Embryo. Mol. Hum. Reprod. 4: 1021-1031.

Park, I., N. Arora, H. Huo, N. Maherali, T. Ahfeldt, A. Shimamura, M.W. Lensch, C. Cowan, K. Hochedlinger, and G.Q. Daley. 2008. Disease-specific induced pluripotent stem cells. Cell 134: 877-886.

Phiel, C.J., F. Zhang, E.Y. Huang, M.G. Guenther, M.A. Lazar, and P.S. Klein. 2001. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant mood stabilizer, and teratogen. J. of Biol. Chem. 276: 36734-36741.

Picou, A.A., R.A. MacLean, B. Dresser, R.A. Godke, and K.R. Bondioli, K.R. 2009. Isolation and characterization of bovine adipose-derived somatic stem cells. Reprod. Fert. Develop. 21:239-240.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S. and D.R.Marshak. 1999. Multilineage potential of adult human mesenchymal stem cells. Science. 284:143-147.

Rideout, W. M., III, K. Eggan and R. Jaenisch. 2001. Nuclear cloning and epigenetic reprogramming of the genome. Science 293: 1093-1098.

Rybouchkin, A., Y. Kato, and Y. Tsunoda. 2006. Role of histone acetylation in reprogramming of somatic nuclei following nuclear transfer. Biol. of Reprod. 74: 1083-1089.

Sell, S. 2005. Adult stem cell plasticity. Stem Cell Rev. 1:1-8.

Stocum, David L., and Gunther K.H. Zupanc. 2008. Stretching the Limits: Stem Cells in Regeneration Science. Developmental Dynamics 237: 3648-3671.

Sun, N., N. J. Panetta, D. M. Gupta, K. D. Wilson, A. Lee, F. Jia, S. Hu, A. M. Cherry, R. C. Robbins, M. T. Longaker and J. C. Wu. 2009. Feeder-Free Derivation of Induced Pluripotent Stem Cells from Adult Human Adipose Stem Cells. Pro. Nat. Acad. Sci. U.S.A. 106: 15720-15725.

Takahashi, K. and Yamanaka, S. 2006. Induction of pluripotent stem cells from embryonic and adult fibroblast cultures by defined cultures. Cell. 126: 663-676.

Takahashi, K., K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, and S. Yamanaka. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131: 861-872.

Thomson, J. A., J. Itskovitz-Eldor, S. S. Shapiro, M. A. Waknitz, J. J. Swiergiel, V. S. Marshall and J. M. Jones. 1998. Embryonic stem cell lines derived from human blastocysts. Science 282: 1145-1147.

Tropel, P., Platet, N., Platel, J.-C., Noël, D., Albrieux, M., Benabid, A.-L. and Berger, F. (2006), Functional neuronal differentiation of bone marrow-derived mesenchymal stem cells. Stem Cells, 24: 2868–2876.

Wang, Y.S., X.R. Xiong. Z.X. An, L.J. Wang, J. Liu, F.S. Quan, S. Hua, and Y. Zhang. 2011. Production of cloned calves by combination treatment of both donor cells and early cloned embryos with 5-aza-2'-deoycytidine and trichostatin A. Theriogenology 75: 819-825.

Wernig, M., A. Meissner, R. Foreman, T. Brambrink, M. Ku, K. Hochedlinger, G. Bernstein, and R. Jaenisch. 2007. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448: 318-325.

Wernig, M., A. Meissner, J.P. Cassady, and R. Jaenisch. 2008. C-Myc is dispensable for direct reprogramming of mouse fibroblasts. Cell Stem Cell 2: 10-12.

West, F.D., S.L. Terlouw, D.J. Kwon, J.L. Mumaw, S.K. Dhara, K. Hasneen, J.R. Dobrinsky, and S.L. Stice. 2009. Porcine induced pluripotent stem cells produce chimeric offspring. Stem Cells and Development 1-28.

Wilmut, I., A. E. Schnieke, J. McWhir, A. J. Kind and K. H. S. Campbell. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature 385: 810-813.

Yang, X., S.L. Smith, X.C. Tian, H.A. Lewin, J. Renard, and T. Wakayama. 2007. Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. Nature Genetics 39: 295-302.

Yoo, B.B., J.C. Cheng, and P.A. Jones. 2004. Zebularine: a new drug for epigenetic therapy. Biochemical Society Transactions 32: 910-912.

Yu, J., M.A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J.L. Frane, S. Tian, J. Nie, G.A. Jonsdottir, V. Ruotti, R. Stewart, I.I. Slukvin, and J.A. Thomson. 2007. Induced pluripotent stem cell lines derived from human somatic cells. Science 318: 1917-1920.

Zhou, H., S. Wu, J.Y. Joo, S. Zhu, D.W. Han, T. Lin, S. Trauger, G. Bien, S. Yao, Y Zhu, G. Siuzdak, H.R. Scholer, L. Duan, and S. Ding. 2009. Generation of induced pluripotent stem cells using recombinant proteins. Cell 4: 381-384.

Zuk, P., Zhu, M., Ashjian, P., Ugarte, D.A., Huang, J.I., Mizuno, H., Alfonzo, Z.C., Frazer, J.K., Benhaim, P. and Hedrick, M.H. 2002. Human adipose tissue is a source of multipotent stem cells. Mol. Biol. Cell. 13:4297-4295.

APPENDIX: PROTOCOLS

Adipose-derived stem cell isolation:

1) Collect adipose tissue from each specimen and place in a wash solution and 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 15-20 mL of 0.25% collagenase solution (0.25% collagenase +1% BSA (Fraction V) + PBS with Ca+ and Mg+
9) Add 200 µL of P/S and 200 µL of Fungizone to the flask containing the tissue and collagenase solution
10) Cover flask with parafilm and place in shake incubator for 2 hours at 200 rpm at 37˚C
11) After incubation, transfer the entire suspension to a new 50 mL tube
12) Centrifuge 300xg for 5 minutes
13) Shake tubes vigorously for 30 seconds
14) Repeat centrifugation
15) Remove tissue from flask, leaving liquid in the tube and shake
16) Pour contents into a double filter syringe with a new 50 mL conical tube receiving the filtered solution
17) Centrifuge at 300 x g for 5 minutes
18) Aspirate collagenase solution and resuspend in 15 mL of PBS with Ca+ and Mg+ +1% BSA (Fraction V)
19) Centrifuge at 300 x g for 5 minutes
20) Aspirate supernatant
21) Add 10 mL of DMEM + 10% FBA + 1% P/S + 2% Fungizone and centrifuge for 5 minutes at 300 x g
22) Aspirate supernatant and resuspend cell pellet in 1 mL of culture medium before placing in a flask
23) Add appropriate amount of culture medium to flask
24) Place in incubator
25) After 24 hours of culture, perform a primary wash and replace with 10 mL of culture medium

Cell Culture:

1) Use a Nalgene filter to filter cell culture medium (89mL DMEM, 10 mL Calf Serum, and 1 mL Pen/Strep)
2) Vacuum out old medium from the flask
3) Wash with 5 mL of PBS without Ca+ and Mg+, then vacuum again
4) Add 0.5mL Trypsin for flask
5) Place the flask in the oven for 5 minutes
6) Add 5 mL of DMEM to flask to inactivate the trypsin. Rinse and suck DMEM about 3 times, and move the liquid to a 15 mL tube for centrifuging
7) Centrifuge at 1200 rpm for 5 minutes
8) Vacuum out the DMEM and Trypsin leaving 1mL in the tube, and suspend the cell pellet in the remaining 1mL
9) Add 90-99 μL of PBS without Ca+ and Mg+ to a tube along with 10-1 μL or cell suspension
10) Add 10 μL in each side of the Hemocytometer to count the cell concentration.

Cell concentration equation = X/2/4 x 1(dilution factor) x 10,000

Amount of cells to add to new flask = amount of cells desired/ amount of cells present x 1,000 = seeding density

Cryopreservation of Mammalian Cells:

1) Perform steps 1-10 of cell culture protocol
2) Determine the desired amount of cells to be frozen (usually no more than 1 million per vial
3) Make enough freezing medium (Calf Serum + 10% DMSO)
4) Centrifuge cells once again at 1200 rpm for 5 minutes
5) Vacuum off medium, and resuspend the pellet in 1mL of freezing medium per vial
6) Place cryo vial in a cooled Mr. Frosty and place at -80˚C for 24 hours before placing in liquid nitrogen

mRNA Isolation from Cultured Cells using Dynabeads Oligo $(dT)_{25}$:

Pre Wash Oligo Dynabeads:

1) Add 250 μL of oligo dynabeads stock to a 1.5 mL tube
2) Put the tube in the magnetic separator for 30 seconds, and then remove the supernatant.
3) Remove the 1.5 mL tube from magnetic separator and add 250 μL of lysis/ binding buffer for washing the beads.
4) Put the tube back into the magnetic separator and remove the lysis/binding buffer.
5) Add 250 μL of lysis/ binding buffer and leave on the beads.

mRNA Isolation from cultured cells:

1) Remove old medium off of the target cells, and add trypsin for 5 minutes.
2) Centrifuge the trypsinized cells along with 5 mL of DMEM to form a cell pellet.
3) Remove supernatant from the pellet, and wash with 1 mL of PBS. Move the solution and resuspended pellet to a 1.5 mL tube.
4) Centrifuge in the little centrifuge for 1200 g (1.2 min-1) for 5 minutes.
5) Remove the supernatant and add 1250 μL of lysis/ binding buffer. Vortex for 10 seconds.
6) Strip the cells with a 21-gauge needle using a 1 mL syringe.
7) Centrifuge the samples at 12000g for 15 seconds.
8) Incubate the samples at room temperature for 10 minutes.
9) Add the pre-washed dynabeads to the sample, and put in the rotator for 10 minutes at room temperature.
10) Place the tube in the magnetic separator for 2 minutes.
11) Remove the supernatant, and wash the beads in 250 μL of buffer A and three times with buffer B.
12) RNA is eluted from the beads by adding 15 μL of water and put in water bath at 70°C for 2 minutes.
13) Put the sample back into the magnetic separator and remove the supernatant, which contains the RNA.

Converting mRNA to cDNA

cDNA Kit (iSCRIPT):

1) Move the 15 μL containing the RNA into a PCR tube.
2) Add to the tube: 4 μL of 5X buffer and 1 μL of reverse transcriptase. If you elute the RNA in 11 μL, then you will need to add 4 μL of nuclease free water.
3) Put the PCR tube in the thermocycler with reverse transcription program
   5 minutes at 25°C
   30 minutes at 42°C
   5 minutes at 85°C
4) After the cycler has finished, the cDNA can be kept in the -80°C freezer.

Treating Bovine ADS cells with VPA or Zeb:

1) Day 1, plate cells with culture medium in 24 well culture dish. Fill 18 wells in a 24 well dish (6 for control, 6 for VPA, and 6 for Zeb). Plate 50,000 cells in each well at passage 3
2) Day 2, change to trt media (6 for control, 6 for VPA, and 6 for Zeb)
3) Change media every 3 days until Day 5 and Day 7, 10, or 14
4) Isolate mRNA using above protocol

Control Culture Medium:

DMEM + 10% FBS + 1% P/S

TRT Culture Media:

1) DMEM +10% FBS + 1%P/S + 100 μM Zeb
2) DMEM + 10% FBS + 1% P/S + 5mM VPA

50 mL of DMEM +10% FBS + 1%P/S + 100 μM Zeb:

500 μL Zeb stock
5 mL FBS
500 μL P/S
44 mL DMEM

50 mL of DMEM +10% FBS + 1%P/S + 5mM VPA:

500 μL VPA stock
5 mL FBS
500 μL P/S
44 mL DMEM

ADS Cells Treated with VPA or Zeb Followed by Transduction with the Lentivirus:

1) Day 1, plate cells with culture medium in 24 well culture dish. Fill 18 wells in a 24 well dish (6 for control, 6 for VPA, and 6 for Zeb). Plate 50,000 cells in each well at passage 3
2) Day 2, change to treatment media (6 for control, 6 for VPA, and 6 for Zeb)
3) Change media every 3 days until Day 5
4) On Day 5, infect the cells (3 wells for each day from each trt) with lentiviral particles
5) 3-4 days later, move to Matrigel coated dish (dishes coated with Matrigel for 1 hour at room temperature before having the Matrigel solution removed)
6) Once the cells from a colony, passage the cells 1-2 more times
7) Collect mRNA after colony formation
8) Isolate mRNA with Dynabeads using above protocol

Producing the Lentiviral Vector:

Day 1:

1) Plate 7x105 HEK-293T cells in 5 mL of cell culture medium in 6 cm tissue culture plate and incubate overnight at 37 C, 5% CO2 overnight

Day 2:

1) Late in the afternoon, in a polypropylene microfuge tube combine 1µg pLKO.1 shRNA plasmid with 750 ng psPAX2 packaging plasmid, 250 ng pMD2.G envelope plasmid, and 2 L serum-free OPTI-MEM
2) Each reaction will need 6 µL FuGENE® + 74µL OPTI-MEM, but add OPTI-MEM first!
3) Incubate the tubes with 100µL volumes for 20-30 minutes at room temperature
4) Add DNA:FuGENE® mix to cells drop wise and swirl to disperse
5) Incubate for 12-15 hours

Day 3:

1) Change the medium to remove the reagent
2) Replace with 5 mL of fresh culture medium
3) Incubate overnight

Day 4:

1) Harvest medium from cells and transfer to polypropylene storage tube
2) Replace with 5 mL of fresh culture medium

Day 5:

1) Harvest medium once again and spin this medium with the medium from Day 4 to pellet any cells that were collected
2) Virus may be stored at -80˚C for long-term

Quantitative RT-PCR:

1) Using a 96 well plate for the reaction, master mixes should be made
2) 10 µL of SsoFast and 6 µL of nuclease free water per reaction should be made into a master mix
3) Then a master mix of primers should be made using 2 µL of reverse and forward primers per reaction
4) Last, the sample or calibrator cDNA needed should be added to each reaction in the 96 well plate before being covered with sealing foil
5) PCR was performed using a LightCycler® 480 Instrument (Roche No. 04729692001).
6) 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 acquisitions per ˚C; and a final holding temperature of 40°C

VITA

Meredith Kathleen Addison was born to Mark and Angie Addison of Monroe, LA. She has two brothers, John and Daniel Addison. She attended Neville High School in Monroe, LA. After high school, she attended Louisiana State University where she pursued a Bachelor's degree in Biological Sciences.

Meredith became interested in pursuing a Master's degree in Animal Sciences her senior year after taking an embryology course in the biology department. She started her program in August of 2009 in Reproductive Physiology, and is now a candidate for a Master's degree in the Department of Animal Sciences at Louisiana State University, Baton Rouge, LA.