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THE GENETIC AND EPIGENETIC EFFECTS OF PRE-TREATMENT WITH THE SMALL MOLECULE INHIBITORS CHIR99021, PD325901, and NuP0178 ON BOVINE FETAL FIBROBLAST CELLS

A Dissertation

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
Doctor of Philosophy

in

The School of Animal Science

by
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B.S., Louisiana State University, 2007
M.S., Louisiana State University, 2011
May 2019
ACKNOWLEDGEMENTS

It is with the greatest appreciation and deepest gratitude that the author acknowledges her major professor, Dr. Kenneth R. Bondioli, for his guidance and support throughout this journey. The pursuit of an advanced degree was made possible by the opportunity, resources, and knowledge he generously provided. However, the completion of an advanced degree was made possible by the patience, motivation, and encouragement he considerately provided.

The author would like to express her sincerest appreciation and thanks to the other members of her graduate committee, Dr. Richard Cooper, School of Animal Sciences, and Dr. Patrick DiMario, Department of Biological Sciences, for their valuable time and contribution to this research. The author would like to extend this gratitude to Dr. Britta Leise, LSU School of Veterinary Medicine, for all of her help in the latter stages of this journey.

The author would also like to thank her fellow graduate students both past and present for their encouragement, help, and advice throughout this time of learning. This appreciation is also extended to the School of Animal Sciences’ faculty and staff.

None of this would be possible without the love and support of the author’s parents, Howard Coley and Ann Coley. Throughout the author’s life, they have encouraged her to always do her best and never give up. Their never-ending love, encouragement, and assurance is sincerely appreciated and will never be forgotten.
Although Howard is longer on this earth, there is no doubt that he is extremely proud of his daughter for this accomplishment.

Lastly, the author would like to thank her husband, Dustin Gaspard, for his unconditional love and support throughout this journey. The road leading to this point has been long and challenging, but Dustin never left her side, and for that, the author is eternally grateful.
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LIST OF ABBREVIATIONS

ASC..........................................................................................Adult Stem Cell
AZA...............................................................................................5-azacytidine
BFF.................................................................................................Bovine Fetal Fibroblast
BMP...............................................................................................Bone Morphological Protein
ChIP..............................................................................................Chromatin Immunoprecipitation
CiPSC..............................................................................................Chemically-induced Pluripotent Stem Cell
CS...............................................................................................Calf Serum
C_{T}............................................................................................Threshold Cycle
C_{TR}............................................................................................Threshold Cycle for the Reference Gene
C_{TT}............................................................................................Threshold Cycle for the Target Gene
DMEM.............................................................................................Dulbecco’s Modified Eagle Medium
DMSO...............................................................................................Dimethyl Sulfoxide
DNMT............................................................................................DNA Methyltransferase
DPBS..............................................................................................Dulbecco’s Phosphate Buffered Solution
DTT.................................................................................................Dithiothreitol
DZNep............................................................................................3-deazaneplanocin
ECC...............................................................................................Embryonal Carcinoma Cell
EDTA..............................................................................................Ethylenediaminetetraacetic Acid
EGC...............................................................................................Embryonic Germ Cell
ESC...............................................................................................Embryonic Stem Cell
FBS...............................................................................................Fetal Bovine Serum
FSK...............................................................................................Forskolin
GFP...............................................................................................Green Fluorescent Protein
GSK-3...........................................................................................Glycogen Synthase Kinase-3
HAT..............................................................................................Histone Acetyltransferase
HDAC...........................................................................................Histone Deacetylase
HDM..............................................................................................Histone Demethylase
HMTase...............................................................................Histone Lysine Methyltransferase
ICM....................................................................................Inner Cell Mass
iPSC..................................................................................Induced Pluripotent Stem Cell
LIF.....................................................................................Leukemia Inhibitory Factor
MEF....................................................................................Murine Embryonic Fibroblast
MEK....................................................................................Mitogen Activated Protein Kinase
mESC...............................................................................Murine Embryonic Stem Cell
MET.....................................................................................Mesenchymal-to-Epithelial Transition
MPF....................................................................................Maturation Promoting Factor
P/S.......................................................................................Penicillin/Streptomycin
PAP.....................................................................................Poly (A) Polymerase
PIC.....................................................................................Protease Inhibitor Cocktail
PMSF..................................................................................Phenylmethylsulfonyl Fluoride
RNAi...................................................................................RNA Interference
RT.........................................................................................Room Temperature
SCNT..................................................................................Somatic Cell Nuclear Transfer
TF.........................................................................................Transcription Factor
TSA.......................................................................................Trichostatin A
VPA......................................................................................Valproic Acid
XEN......................................................................................Extraembryonic Endoderm
The ability to produce genetically superior livestock has established somatic cell nuclear transfer (SCNT) as an invaluable tool in commercial livestock production. Successful reprogramming of somatic cells towards pluripotency requires the epigenetic marks characteristic of the differentiated cell type first be erased in order to inactivate the somatic cell program and activate the embryonic program. Several small molecules have been shown to improve both the kinetics and efficiency of reprogramming. These chemical modifiers aid in overcoming the “roadblocks” encountered during the reprogramming process by inducing the necessary epigenetic modifications needed to silence the somatic cell genome and completely reactivate the embryonic stem cell (ESC) genome. If small molecules are used to “prime” the somatic cells to be used as donor cells in SCNT, the efficiency of nuclear reprogramming during SCNT may be enhanced.

We first assessed the effect of pre-treatment with small molecules on the expression of Oct-4, Nanog, and Sox-2 in bovine fetal fibroblast (BFF) cells. Chemical treatment consisted of 3 small molecules: PD0325901, a mitogen activated protein kinase/ERK kinase (MEK) inhibitor; CHIR99021, a glycogen synthase kinase-3 (GSK3) inhibitor; and NuP0178, a G9a histone methyltransferase inhibitor. No significant difference in transcript levels for Oct-4, Nanog, or Sox-2 was detected, indicating that this combination of small molecule inhibitors does not have an effect on the expression of Oct-4, Nanog, and Sox-2 in BFF cells.
We next sought to assess the effects this combination of small molecule inhibitors has on the epigenetic state of Oct-4, Nanog, and Sox-2 in BFF cells. Chromatin Immunoprecipitation was used to quantify the enrichment of key histone modifications on the promoter regions of Oct-4, Nanog, and Sox-3 in BFF cells treated with and without PD0325901, CHIR99021, and NuP0178 over time. Time, treatment, and a time*treatment interaction were found to have a significant effect on the histone modifications analyzed. Determining how the expression of these factors alters the epigenetic marks in the promoter regions of key pluripotency-associated genes will allow for the development of defined conditions which best mimic the epigenetic landscape of ESC, ultimately leading to engineering the ideal donor cell for successful SCNT.
CHAPTER I
INTRODUCTION

In 2006, Takahashi and Yamanaka reported the generation of a population of cells similar to embryonic stem cells (ESC) by transfecting mouse tail fibroblast cells with four transcription factor-encoding genes. These cells, termed induced pluripotent stem cells (iPSC), are morphologically similar to pluripotent ESC and, most importantly, demonstrate key characteristics of pluripotent ESC, including expressing stem cell markers, forming teratomas containing cells of all three germ layers, and contributing to multiple cell lineages (Takahashi and Yamanaka, 2006). This landmark discovery paved the way for a rapidly progressing field of research.

The resetting of the somatic cell nucleus to an embryonic state involves several complex mechanisms. The expression of Oct-4, Nanog, Sox-2 and other pluripotency-associated genes is silenced through the differentiation-induced actions of DNA methylation, histone deacetylation and histone methylation. These repressive mechanisms that function to silence the embryonic program must be overcome during reprogramming (Hochedlinger and Plath, 2009). These key developmental genes contain bivalent domains consisting of both inhibitory H3K27 methylation marks and activating H3K4 methylation marks. These bivalent domains are lost upon cellular differentiation, indicating that they play an important role in maintaining pluripotency. In addition, epigenetic silencing associated with H3K9 methylation also contributes to the maintenance of pluripotency in ESC. H3K9me3 is associated with repressive chromatin, and is maintained at low levels in ESC (Bernstein et al., 2006). These marks are a key feature in the epigenetic signature of ESC.
Studies have revealed that epigenetics play a crucial role in nuclear reprogramming. Resistance to reprogramming is largely attributed to the phenomenon of epigenetic memory that cells retain throughout numerous cell divisions. As cells become more differentiated, they acquire epigenetic marks that make their nuclei increasingly resistant to reprogramming (Pasque et al., 2011). However, modulations of the epigenetic processes that accompany nuclear reprogramming may facilitate the conversion to an epigenetic landscape that is more permissive to reprogramming. Various tools have been employed to help cells overcome this reprogramming roadblock, including the use of synthetic chemical epigenetic modulators known collectively as “small molecules”. Several chemical compounds that control epigenetic enzymes, such as HDAC, HMT, DNMT, and histone demethylase (HDM) have been shown to improve reprogramming efficiency, or even replace the need to use certain transcription factors (Zhang et al., 2012). This is especially promising for the clinical application of iPSC as there are serious concerns regarding the safety of current genetic approaches to nuclear reprogramming, as well as traditional culture systems that are used to maintain iPSC. Small molecules provide an attractive approach to addressing these challenges, as they offer a number of compelling advantages. First, the biological effects of small molecules are typically rapid, reversible and dose-dependent, allowing precise control over specific outcomes by fine-tuning their concentrations and combinations. Second, the structural diversity that can be provided by synthetic chemistry allows the functional optimization of small molecules. Third, compared with genetic interventions, the relative ease of the handling and
administration of small molecules make them more practical for in vitro and in vivo applications, and for further therapeutic development (Zhang et al., 2012).

Once established, cellular identity is remarkably stable. Chromatin modifications are faithfully inherited from cell division to cell division, highlighting the major hurdles iPSC must overcome in order to be fully reprogrammed to pluripotency. The use of small molecule compounds that target chromatin modifications and/or specific signaling pathways have proven to be effective at overcoming these reprogramming hurdles. In the present study, we sought to assess the genetic and epigenetic effects pre-treatment with a small molecule cocktail have on BFF cells.
CHAPTER II
LITERATURE REVIEW

The History of Nuclear Reprogramming

In 2006, Takahashi and Yamanaka reported the generation of a population of cells similar to ESC by transfecting mouse tail fibroblast cells with four transcription factor-encoding genes. These cells, termed induced pluripotent stem cells (iPSC), are believed to be immensely beneficial in the study and treatment of disease as the direct reprogramming of somatic cells provides an opportunity to generate patient- or disease-specific pluripotent stem cells (Nakagawa et al., 2008). The supposed potential of iPSC lies in the fact that these cells are morphologically similar to pluripotent ESC and, most importantly, demonstrate key characteristics of pluripotent ESC, including expressing stem cell markers, forming teratomas containing cells of all three germ layers, and contributing to multiple cell lineages (Takahashi and Yamanaka, 2006). This landmark discovery has opened a new frontier in the field of regenerative medicine because, for the first time, a realistic way of generating sufficient numbers of patient-specific pluripotent stem cells exists (Amabile and Meissner, 2009). Furthermore, this method of generating iPSC has provided researchers with a new and unique tool to study mammalian development and the mechanisms underlying nuclear reprogramming.

The breakthrough discovery that lineage-restricted somatic cells can be reprogrammed to a pluripotent state through the ectopic expression of defined transcription factors represents the culmination of over 50 years of research. The history of nuclear reprogramming begins in 1952 with Briggs and King. The long-standing question of whether the process of development and cell differentiation
irreversibly alters the genome prompted Briggs and King (1952) to develop a system to directly test the genetic equivalence of somatic cell nuclei and embryonic cell nuclei. In this first published nuclear transfer study, Briggs and King (1952) reported generating cloned frogs by transferring nuclei isolated from late-stage embryos into enucleated oocytes. This work, together with seminal studies by Gurdon (1962; Gurdon et al., 1975), provided the first conclusive evidence that genes of somatic cells are not irreversibly changed during differentiation. Furthermore, these studies demonstrated the ability of the oocyte cytoplasm to facilitate the de-differentiation of the somatic cell nuclei in order to reset the developmental program (Briggs and King, 1952; Gurdon, 1962; Gurdon et al., 1975).

Despite the early success of nuclear transplantation in frogs, it was not until 1997 that the first mammal was successfully cloned using somatic cell nuclear transfer (SCNT). The birth of “Dolly”, a cloned sheep produced by fusing a mammary cell that had been induced to enter a quiescent state with an enucleated oocyte (Wilmut et al., 1997), marks one of the most celebrated events in the history of biological research. Heralded by Science magazine as “the breakthrough of 1997”, “Dolly” decisively proved that even a fully differentiated mammalian somatic cell can be reverted to an embryonic state when transferred into an enucleated oocyte (Wilmut et al., 1997). In spite of a 45 year gap between the first nuclear transfer experiment by Briggs and King (1952) and the birth of “Dolly”, many key discoveries in the field of nuclear reprogramming were made during this 45 year period, including the development of different techniques that allowed researchers to derive, culture and study pluripotent cell lines. Scientists’ earliest insight into the nature of pluripotent cells came from studying embryonal
carcinoma cells (ECC). Derived from germ cell tumors known as teratocarcinomas, researchers discovered that ECC could be clonally expanded in culture while remaining pluripotent (Finch and Ephrussi, 1967). The establishment of these immortal pluripotent cell lines marks a key event in the history of nuclear reprogramming because it provided scientists with a tool to explore the regulatory mechanisms underlying pluripotency. Among these scientists were Miller and Ruddle, who showed that the hybrid cells resulting from the fusion of murine teratocarcinoma cells and thymus cells adopted the biochemical and development properties of their ECC parent (Miller and Ruddle, 1976). The dominance of the pluripotent state over that of the somatic cell suggested that gene expression is not only regulated by cis-acting DNA elements but also by trans-acting factors present in ECC that confer pluripotency in somatic cells upon fusion (Stadtfeld and Hochedlinger, 2010). Short-lived, non-dividing, multinucleate fusion products of two distinct cell types, known as heterokaryons provided the first definitive evidence that previously silent genes typical of diverse differentiated mammalian cells could be activated in other differentiated cell types (Blau et al., 1983). Because a proliferating population of hybrid cells often results in nuclear fusion, loss and rearrangement of chromosomes, gene activation as a result of nuclear reprogramming cannot accurately be assessed. Heterokaryons, on the other hand, do not divide; therefore, the nuclei of the two cells remain distinct and intact (Yamanaka and Blau, 2010). In the first heterokaryon study, human amniotic cells and murine muscle cells were fused together to form heterokaryons that expressed several muscle proteins, indicating that muscle genes had been activated in non-muscle cells (Blau et al., 1983). The belief that previously silent genes could be activated in muscle-cell-containing heterokaryons was
corroborated by other groups soon after (Wright, 1984), and it quickly became apparent that this was true for other cell types as well.

The results of these heterokaryon experiments lead researchers to the make the same conclusion about mammalian somatic cells as Briggs and King (1952) and Gurdon (1962; Gurdon et al., 1975) did about amphibian somatic cells years before: the differentiated state of somatic cells is not fixed and irreversible. Instead, these experiments illustrated that the generally stable state of differentiated somatic cells can be altered in response to changes in the cells’ environment, lending support to the theory of cellular plasticity. Researchers hypothesized that the differentiated state of somatic cells is dictated by the balance of regulators and requires continuous regulation. Alterations in the cells’ environment induce perturbations in the stoichiometry of these regulators, allowing the fate of somatic cells to change (Blau et al., 1983). These observations prompted researchers to further investigate the regulators responsible for maintaining the differentiated state of somatic cells and how changes in the levels of these regulators in the cell can alter cell fate. In 1987, Schneuwly et al. demonstrated that the overexpression of a D. melanogaster homeotic gene, Antennapedia, under the control of a heat-shock gene promoter led to the formation of an additional set of legs instead of an antenna. Around the same time, the first mammalian master regulatory transcription factor, MYOD, was identified. Davis et al. (1987) reported the formation of myofibers in murine fibroblast cell lines transduced with retroviral vectors expressing the skeletal muscle transcription factor MYOD, demonstrating that overexpression of this single transcription factor is sufficient to convert fibroblast to myoblast. These studies demonstrated that lineage-associated
transcription factors—which help to establish and maintain cellular identity during development by driving the expression of cell type-specific genes while suppressing lineage-inappropriate genes—can change cell fate when ectopically expressed in certain heterologous cells (Stadtfeld and Hochedlinger, 2010). Importantly, these studies prompted a more systematic search for transcription factors that could induce the conversion of differentiated cells to a pluripotent state. Almost 20 years later, these factors were identified (Takahashi and Yamanaka, 2006).

Nuclear Reprogramming Strategies

Each of the aforementioned experiments represents a major milestone in the history of nuclear reprogramming. These key discoveries have impacted the field of nuclear reprogramming by providing scientists’ not only with a better understanding of the mechanisms underlying the process of nuclear reprogramming but also with the conceptual framework needed for the development of current reprogramming techniques. Currently, there are four methods of reprogramming differentiated somatic cells to a pluripotent state: SCNT (Wilmut et al., 1997), fusion with ESC (Cowan et al., 2005), exposure to ESC extracts (Bru et al., 2008), introduction of defined factors (Takahashi and Yamanaka, 2006).

Somatic Cell Nuclear Transfer

In 1997, Wilmut and colleagues introduced the world to “Dolly”, the first successfully cloned mammal from an adult cell. By fusing a mammary epithelial cell from a Finn Dorset ewe with an enucleated donor oocyte, Wilmut et al. (1997) unknowingly created “the world’s most famous sheep”. Since the birth of “Dolly”, a wide
range of species have been successfully cloned using SCNT, including domesticated animals such as the cow (Kato et al., 1998) and dog (Lee et al., 2005), as well as wild animals like the African wildcat (Gomez et al., 2004) and wolf (Kim et al., 2007).

Wilmut and colleagues succeeded at what had been deemed for many years as “biologically impossible”. The birth of “Dolly”, however, provided clearly convincing evidence that the nucleus of even fully differentiated mammalian cells can be reprogrammed to an embryonic state when transferred into enucleated oocytes (Wilmut et al., 1997). Wilmut and colleagues succeeded at what others had been attempting for years by implementing cell cycle synchronization into their strategy. They theorized that inducing the donor cells to exit the cell cycle by serum deprivation would change the chromatin structure in such a way that was conducive to nuclear reprogramming.

Under normal conditions in vivo, high levels of Maturation Promoting Factor (MPF) found in the cytoplasm of metaphase II (MII) oocytes induce nuclear envelope breakdown and chromosome condensation to facilitate entry into the M phase of the cell cycle. The activity of MPF can have beneficial or harmful effects upon the reconstructed embryo depending on the cell cycle stage of the donor cell (Campbell et al., 1993; Fulka et al., 1998). High levels of MPF in the MII oocyte cause nuclear envelope breakdown and chromosome condensation in the transferred nucleus, irrespective of cell cycle stage of the donor cell. If the nucleus is in S or G2 phase, then the potential for reduplication of the genome directed by the recipient cytoplasm will result in aberrant development (Hanocq-Quertier et al., 1976; Sunkara et al., 1979; Campbell et al., 1996; Kikyo and Wolffe, 2000). If DNA synthesis is not yet complete, initiation of premature nuclear envelope breakdown and chromosome condensation will
lead to chromosome loss and aneuploidy (Kikyo and Wolffe, 2000). To facilitate proper nuclear remodeling by MPF, the donor nuclei should be in G1 phase or G0 phase, in which the cells remain metabolically active but have exited the cell cycle. Donor nuclei in G1 or G0 phase transferred to recipient oocytes containing high levels of MPF will condense normally and maintain correct ploidy of subsequent embryos at the end of the first cycle (Campbell et al., 1993; Macháty et al., 2002). The usefulness of quiescent cells has been attributed to their reduced transcriptional activity and chromatin modifications that are associated with cells in G0, which may enhance their epigenetic plasticity (Armstrong et al., 2006).

A number of critical processes must occur and a temporal pattern of events will have to be obeyed in order to achieve reprogramming of the somatic cell nucleus by SCNT. These processes include transcriptional silencing of the donor nucleus, erasure of differentiated cellular memory, appropriate activation of the reconstructed “one-cell embryo”, and appropriate embryonic gene expression at all later stages of development (Santos and Dean, 2004). This cascade of reprogramming of events is initiated by the exchange of proteins that occurs when a somatic cell donor nucleus is transplanted into an enucleated oocyte. The first indication of nuclear reprogramming is the breakdown of the somatic cell nuclear envelope. As discussed above, nuclear envelope breakdown is associated with high MPF levels. Soon thereafter, the somatic cell chromosomes begin to condense. All of the factors necessary for reprogramming the somatic cell nuclei back to a totipotent state are present in the MII oocyte cytoplasm. Researchers have been exploring these reprogramming factors since Wilmut et al. (1997) first reported the successful production of a cloned sheep, and although more than a decade
has passed, they remain largely unknown. Some proteins have been identified, including nucleoplasmin and N1/N2. Both of these molecular chaperones can mediate the transfer of core histones to DNA and the assembly of nucleosomes. Nucleoplasmin plays a role in the exchange of the somatic linker histone variants (H1, H1') for oocyte-specific histone variant B4 and the chromatin structural protein HMG1 (Dimitrov and Wolffe, 1996).

Since the birth of Dolly, viable NT-derived offspring have been produced in many species ranging from domesticated animals such as dogs and goats, to wild animals such as African wildcats and wolves (Thuan et al., 2010). Nevertheless, the overall efficiency of SCNT is between 0-3% (number of live offspring as a percentage of the number of nuclear transfer embryos) (Paterson et al., 2003). The majority of cloned embryos die in utero, and those embryos that do develop to term often exhibit a variety of developmental abnormalities. Placental abnormalities are a common feature among NT embryos and account for the high incidence pregnancy loss associated with NT embryos. It is thought that defects in placental function are also responsible for many of the developmental abnormalities in cloned fetuses, including Large Offspring Syndrome (Yang et al., 2007).

While a great deal of the attention “Dolly” garnered stemmed from intense public debate over ethical concerns regarding cloning, many in the agricultural, scientific and medical community were excited over the potential applications of SCNT. SCNT may be used to produce multiple copies of a genetically superior farm animal with proven performance. Additionally, SCNT may be used to preserve endangered species. Given that somatic cells are easily obtained from adult animals, cultured in the laboratory and
then genetically modified, cloning procedures are ideal for introducing specific genetic modifications in farm animals. Transgenic animal production provides numerous opportunities not only for agriculture, but for biomedicine as well, as these animals can be used for the production of pharmaceutical proteins or xenotransplantation (Whyte and Prather, 2011). In recent years, however, the potential capabilities of SCNT have been overshadowed by ethical concerns as well as the inefficient nature of the procedure, leading researchers to explore alternative reprogramming methods for the generation of ESC-like cells.

Fusion with ESC

Cell fusion studies date back to the 1960’s. These pioneering experiments provided novel evidence that gene expression is regulated not only by cis-acting DNA elements but also by trans-acting repressors (Harris et al., 1969; Miller and Ruddle, 1976; Weiss et al., 1977). Approximately a decade later, cell fusion studies offered the first definitive evidence that the differentiated state of mammalian somatic cells is not fixed and irreversible but, instead, is dictated by the balance of regulators and requires continuous regulation (Blau et al., 1983; Blau et al., 1985; Blau and Baltimore, 1991). Such studies could not be taken further until recent molecular technologies were developed, at which point cell fusion experiments showed that the pluripotent state can dominate over the differentiated state under certain conditions, resulting in the activation of previously silenced genes (Yamanaka and Blau, 2010). Tada and colleagues (1997) reported the first successful reprogramming of somatic cells in proliferative hybrids after they fused female EGC with thymocytes from an adult male. They continued their cell fusion experiments, and in 2001 reported that thymocytes were reprogrammed to a
pluripotent state after being fused with mouse ESC (mESC). 48 hours after fusion with mESC, Oct-4 was reactivated in hybrid cells, indicating pluripotency had been reestablished. This was also testified by the hybrids' contribution to all three germ layers as well as the epigenetic status as assessed by DNA methylation patterns of imprinted and non-imprinted genes (Tada et al., 2001). Rathjen and colleagues (2002) produced similar results when they fused mouse ESC-derived neuroectoderm with undifferentiated mouse ESC, resulting in hybrid cells that expressed pluripotency-associated genes at levels comparable to those in ESC. To test if human ESC were capable of nuclear reprogramming like their murine counterpart, Cowan et al (2005) fused human ESC with human fibroblast cells. Following fusion, hybrid cells containing both ESC and fibroblast cell chromosomes in a single nucleus were formed; however, the hybrid cells exhibited characteristics consistent with the ESC, indicating that the phenotype of human ESC is dominant and that they are also capable of reprogramming human somatic cells (Cowan et al., 2005). Furthermore, DNA analysis showed that the promoter region of Oct-4 in the hybrid cells was demethylated and indistinguishable from that found in human ESC, proving that the epigenetic information controlling the transcription of pluripotency genes was reprogrammed (Cowan et al., 2005).

Unlike the ooplasm of an enucleated oocyte in SCNT, nuclear reprogramming of a somatic cell to a less differentiated state through cell fusion is governed by ESC nuclei, which reactivates pluripotency-associated genes and silences the gene expression of the somatic cell. When the nucleus and the cytoplasm of mESC were fused with neurosphere cells, only karyoplasts, not cytoplast, could reactivate Oct-4 in the somatic genome (Do and Schöler, 2004). This is consistent with cloning
experiments in amphibians and mice, which indicate that successful reprogramming depends on direct injection of nuclei into the MII oocyte, where nuclear factors are available in the cytoplasm (Hochedlinger and Jaenisch, 2006).

Cell fusion is an attractive approach to studying pluripotency and the regulatory mechanisms involved in nuclear reprogramming. Although it is well established that somatic cells can be reprogrammed to a pluripotent state through fusion with various pluripotent cell types, cell fusion for the generation of pluripotent cells for clinical or therapeutic application is not best. Hybrid cells contain an abnormal ploidy as well as nonautologous genes from the pluripotent parent, which could result in immune rejection (Pralong et al., 2006). Removal of the pluripotent genome must be achieved before the hybrid cells can be used clinically.

Exposure to ESC Extracts

Cell fusion experiments involving the fusion of pluripotent cell types with various somatic cell types provided critical insight of the molecular regulators of nuclear reprogramming. The work of Tada and colleagues (1997, 2001) as well as Cowen and colleagues (2005) showed that pluripotent cells can trigger epigenetic reprogramming to a less differentiated state in a variety of somatic cell types after cellular fusion, proving that pluripotent cell types harbor dominant reprogramming activities. It was experiments like these that provided the inspiration for the development of a cell-free means to reprogramming the nuclei of somatic cells to a pluripotent state. One such way of doing this is through exposure to ESC extracts. Ha°kelien et al (2002) had previously demonstrated the reprogramming abilities of cellular extracts using human 293T
fibroblast cells and human T cells. Ha°kelien et al (2002) derived nuclear and cytoplasmic extracts from human peripheral blood T cells by lysing the cells using a sonicator. For the 293T cells to take up the T cell extracts, their cellular membrane was permeabilized using Streptolysin O (SLO), which binds to cholesterol in the cell membrane and forms holes in the plasma membrane of the cell. The T cell extracts and the permeabilized 293T cells were incubated together for approximately 50 min and then the plasma membrane was resealed using a CaCl$_2$-containing cocktail (Ha°kelien et al., 2002). Plasma membrane resealing following SLO exposure is dependent upon Ca$^{+2}$, as the Ca$^{+2}$ influx triggers a rapid repair process to the permeabilized plasma membrane (Walev et al., 2001).

Using the same method of extract derivation and membrane permeabilization as described by Ha°kelien et al (2002), Taranger and colleagues (2005) demonstrated that extracts from both undifferentiated ECC and ESC can induce somatic cells to dedifferentiate and, in turn, acquire pluripotent characteristics. 293T cells exposed to human teratocarcinoma NCCIT cell extracts and mESC extracts formed defined colonies that were maintained for an extended period of time in culture. Gene expression profiling revealed a dynamic up-regulation of genes associated with undifferentiated cell types, parallel with down-regulation of 293T cell genes and other genes associated with differentiated cell types. Pluripotency-associated genes Oct-4, Nanog, and Sox-2 were among the genes up-regulated in 293T cells exposed to extracts of undifferentiated cell types. Bisulfite sequencing revealed that the Oct-4 promoter was demethylated in extract-treated 293T cells, suggesting that NCCIT and mESC extracts are capable of eliciting Oct-4 demethylation in somatic cells.
Additionally, extract-treated 293T cells demonstrated multilineage differentiation capabilities by giving rise to cell types of the three germ layers (Taranger et al., 2005).

In an effort to better understand the molecular processes underlying nuclear reprogramming, researchers built upon this study and were able to provide evidence of reprogramming of DNA methylation and histone modifications on the Nanog promoter and throughout the Oct-4 regulatory region in human epithelial cells (Freberg et al., 2007). In order to identify those cells that had been stably reprogrammed to express pluripotency-associated genes, the treated cells in all of the aforementioned studies had to be grown in culture for several weeks, which makes it difficult to ascertain reprogramming efficacy as well as complicating further biochemical analysis procedures (Bru et al., 2008). In 2008, however, Bru et al. reported the detection of key pluripotency-associated genes in ESC-extract treated cells within a few hours of exposure, proving the first stages of reprogramming do not require a long incubation period. Interestingly, the expression of these genes increased in the 48 hours following exposure to extracts, indicating that long-term reprogramming of gene expression had been induced (Bru et al., 2008). While further studies to validate extract-based nuclear reprogramming are needed, it has proven to be an effective means to nuclear reprogramming, which may be a more appealing option of generating iPSC than by either SCNT or cell fusion.

Nuclear reprogramming techniques are believed to be immensely beneficial in the study and treatment of disease as the direct reprogramming of somatic cells provides an opportunity to generate patient- or disease-specific pluripotent stem cells (Nakagawa et al., 2008). While all four experimental techniques have demonstrated the
ability to successfully reprogram gene expression and promote pluripotency in terminally differentiated cells (Thomson et al., 1998; Cowan et al., 2005; Takahashi and Yamanaka, 2006; Bru et al., 2008), not all of the methods are suitable for generating iPSC for clinical use. Aside from the obvious ethical issues regarding nuclear reprogramming by SCNT and fusion with ESC, major technical obstacles impede their clinical applications. The inefficient nature of SCNT, coupled with the fact that it is a technically challenging procedure, make it unlikely that SCNT could be performed on a large scale to derive pluripotent cell lines routinely for every patient (Amabile and Meissner, 2009). Although nuclear reprogramming by fusion with ESC circumvents these hindrances, abnormal ploidy and the presence of nonautologous genes from the pluripotent parent cells prevent the therapeutic use of pluripotent hybrid cells (Pralong et al., 2006). For this approach to be viable, a practical means of removing the nucleus of the ESC from the hybrid cell must be developed in order to generate diploid customized cells for transplantation therapy. It will be difficult, if not impossible, to selectively eliminate the entire set of ESC chromosomes from the hybrid cells if future research determines DNA replication is required for reprogramming (Hochedlinger and Jaenisch, 2006).

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

Defined Factors

One of the most significant breakthroughs in stem cell research to occur in recent years is the production of iPSC by defined factors. Indeed, it is arguably the most celebrated scientific advancement since the birth of “Dolly”. Like the previously mentioned methods of generating iPSC, the ectopic expression of defined transcription factors can also generate genetically-tailored stem cells for therapeutic use by reprogramming the nucleus of a differentiated cell to function like that of an ESC. In contrast to the production of iPSC by SCNT, fusion with ESC or exposure to ESC extracts, the defined factors methodology is far less controversial because it does not make use of ESC or oocytes. Instead, this technique relies upon the actions of four transcription factors to elicit pluripotent cells from otherwise developmentally-restricted cells. Considering the transcription factors known to function in the maintenance of
pluripotency in ESC as well as those specifically expressed in ESC, Takahashi and Yamanaka (2006) selected 24 genes as contenders for factors capable of inducing pluripotency in somatic cells. To determine which genes are critical for reprogramming a somatic cell to an ESC-like state, Takahashi and Yamanaka (2006) developed an assay system in which a gene's ability to induce pluripotency was determined by its ability to trigger the formation of G418-resistant colonies following its induction to mouse embryonic fibroblast cultures by retroviral transduction. Of these 24 genes, Oct-4, Sox-2, c-Myc and Klf-4 were identified as the essential factors for generating iPSC directly from fibroblast culture (Takahashi and Yamanaka, 2006). The resultant iPSC exhibited morphological features and proliferative properties consistent with ESC, and also expressed ESC marker genes. Furthermore, subcutaneous injection of the iPSC in nude mice elicited the formation of teratomas that contained tissues from all three germ layers, indicative of the pluripotent state of the iPSC (Takahashi and Yamanaka, 2006). The crucial roles Oct-4 and Sox-2 play in maintaining ESC identity are well established; therefore, it was not surprising to discover they also serve in the direct production iPSC in culture. However, it was surprising to find that Nanog, whose expression is essential to the pluripotent-state of ESC, was dispensable to this process, whereas c-Myc and Klf-4 were imperative (Takahashi and Yamanaka, 2006). c-Myc and Klf-4 are proposed to indirectly enhance Oct-4, Sox-2 and Nanog function, respectively, in the iPSC. It is believed that c-Myc may induce global histone acetylation in the mammalian genome (Fernandez et al., 2003), which, in turn, potentiates Oct-4 and Sox-2 target binding. Klf-4, on the other hand, may contribute to Nanog activation by repressing p53, which is known to suppress Nanog expression in ESC during differentiation (Lin et al., 2005).
Indeed, the exact mechanisms these factors employ to induce pluripotency in somatic cells is undetermined, however, their ability to do so is universal. Although human ESC differ from murine ESC in many aspects, Takahashi et al. (2007) reported the generation of iPSC from adult human fibroblast cultures using the same defined factors less than a year after their initial study. The functional uniformity of Oct-4, Sox-2, c-Myc and Klf-4 across species suggests that these four factors are fundamentals of a common pluripotency-governing system. Furthermore, the similarities in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, telomerase activity, and pluripotent differentiation ability noted between human iPSC and true human ESC (Nakagawa et al., 2008) are similar to those observations between murine iPSC and murine ESC (Takahashi and Yamanaka, 2006).

**Epigenetic Reprogramming**

In all of the aforementioned reprogramming strategies, differentiated somatic cells are reverted to an ESC-like state as a result of changes in the epigenome. Although epigenetic modifications are heritable, all four reprogramming methodologies have demonstrated that these modifications can be experimentally reversed. Epigenetic changes are heritable modifications to DNA or chromatin that allow differentiated cells to perpetuate the molecular memory needed to retain their identity (Tada et al., 1997; Jones and Takai, 2001). The principle function of epigenetic modifications is to regulate the repression of genes not required in specific cell types at specific stages of development without changing DNA sequence (Wolffe and Matzke, 1999).
Successful reprogramming of somatic cells towards pluripotency requires the epigenetic marks characteristic of the differentiated cell type first be erased in order to inactivate the somatic cell program and activate the embryonic program. Recent studies reporting the activation of the embryonic marker SSEA 1 following the downregulation of somatic markers, such as Thy 1 and collagens, provide evidence in support of the notion that silencing the somatic cell program is an important initial step in re-establishing pluripotency (Brambrink et al., 2008; Stadtfeld et al., 2008). The activation of SSEA 1 in reprogrammed fibroblast cells is an especially important intermediate stage of transcription factor-induced reprogramming because it promotes the gradual reactivation of other markers associated with pluripotency, including Oct-4, Nanog, Sox-2, telomerase and the silent X chromosome in female fibroblasts (Brambrink et al., 2008; Stadtfeld et al., 2008).

The expression of Oct-4, Nanog, Sox-2 and other pluripotency-associated genes is silenced through the differentiation-induced actions of DNA methylation, histone deacetylation and histone methylation. These repressive mechanisms that function to silence the embryonic program must be overcome during reprogramming (Hochedlinger and Plath, 2009). Regardless of the methodology employed, nuclear reprogramming is associated with a global reversal of DNA methylation. Both active and passive mechanisms of DNA demethylation have been proposed to occur in reprogramming, but the precise nature of the mechanisms underlying nuclear reprogramming remains largely unknown. It is possible that reprogramming by defined factors facilitates the direct binding of these factors to promoter or enhancer regions of target sites, which interfere with the methylation of newly synthesized DNA during DNA replication.
Similarly, passive demethylation could also occur as a result of stochastic impairments to specific factors known to contribute to the stable inheritance of methylation patterns, thus inhibiting Dnmt-1 function indirectly (Hochedlinger and Plath, 2009).

Inefficient reprogramming in SCNT embryos has been linked to flaws in the demethylation process. This may in part be due to the fact that the somatic nuclei contain the somatic form of Dnmt-1, which, unlike the oocyte form, is capable of maintaining methylation levels (Reik et al., 2001). Alternatively, DNA methylation could be actively removed by the recruitment of a demethylating enzyme, although the presence of demethylation enzymes in mammalians is still under debate (Hochedlinger and Plath, 2009).

**Small Molecules**

The notion that the aforementioned repressive mechanisms that function to silence the embryonic program actions must be overcome in order for successful nuclear reprogramming to occur has prompted the use of various chemical inhibitors involved in DNA methylation, histone deacetylation and histone methylation. Such chemical inhibitors aid in overcoming the “roadblocks” encountered during the reprogramming process by inducing the necessary epigenetic modifications needed to silence the somatic cell genome and completely reactivate the ESC genome. Several small molecules, affecting specific signaling pathways and/or chromatin modifications, have been shown to improve both the kinetics and efficiency of reprogramming (summarized in Table 2.1)(Zhang et al., 2012). In addition, pre-treatment of cells with
Table 2.1. Known compounds that modulate stem cell fate and reprogramming

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Identity</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valproic acid (VPA)</td>
<td>HDAC inhibitor</td>
<td>Promotes MEF reprogramming efficiency, and enables Oct4- and Sox2-mediated reprogramming of human fibroblasts;</td>
<td>(Huangfu et al., 2008a; Huangfu et al., 2008b)</td>
</tr>
<tr>
<td>VPA</td>
<td>HDAC inhibitor</td>
<td>facilitates proteins mediated reprogramming of MEFs</td>
<td>(Zhou et al., 2009)</td>
</tr>
<tr>
<td>Suberoylanilide hydroxamic acid (SAHA)</td>
<td>HDAC inhibitor</td>
<td>Promotes MEF reprogramming efficiency</td>
<td>(Huangfu et al., 2008a)</td>
</tr>
<tr>
<td>Trichostatin A (TSA)</td>
<td>HDAC inhibitor</td>
<td>Promotes MEF reprogramming efficiency</td>
<td>(Huangfu et al., 2008a)</td>
</tr>
<tr>
<td>Sodium butyrate (NaB)</td>
<td>HDAC inhibitor</td>
<td>Enhances reprogramming efficiency of human adult or fetal fibroblasts;</td>
<td>(Mali et al., 2010)</td>
</tr>
<tr>
<td>NaB</td>
<td>HDAC inhibitor</td>
<td>facilitates Oct4-only mediated reprogramming when combined with A-83-01/PD0325901/PS48</td>
<td>(Zhu et al., 2010)</td>
</tr>
<tr>
<td>BIX-01294</td>
<td>G9a HMT inhibitor</td>
<td>Enables NPC reprogramming mediated by Oct4 and Klf4, or substitutes for Oct4 in NPC reprogramming;</td>
<td>(Shi et al., 2008b)</td>
</tr>
<tr>
<td>BIX-01294</td>
<td>G9a HMT inhibitor</td>
<td>promotes MEF reprogramming mediated by Oct4 and Klf4</td>
<td>(Shi et al., 2008a)</td>
</tr>
<tr>
<td>RG108</td>
<td>DNMT inhibitor</td>
<td>Promotes MEF reprogramming mediated by Oct4 when combined with BIX-01294</td>
<td>(Shi et al., 2008a)</td>
</tr>
<tr>
<td>Parnate</td>
<td>LSD1 inhibitor</td>
<td>Enables reprogramming of human keratinocytes mediated by Oct4 and Klf4;</td>
<td>(Li et al., 2009b)</td>
</tr>
<tr>
<td>Parnate</td>
<td>LSD1 inhibitor</td>
<td>facilitates the conversion of mEpiSCs to naïve pluripotent state</td>
<td>(Zhou et al., 2010)</td>
</tr>
<tr>
<td>Compound name</td>
<td>Identity</td>
<td>Function</td>
<td>References</td>
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</tr>
<tr>
<td>PD0325901</td>
<td>MEK inhibitor</td>
<td>Blocks differentiation pathway of ESCs and supports self-renewal;</td>
<td>(Ying et al., 2008; Tsutsui et al. 2011)</td>
</tr>
<tr>
<td>PD0325901</td>
<td>MEK inhibitor</td>
<td>supports ESC derivation from refractory strains or species;</td>
<td>(Nichols et al., 2009; Buehr et al., 2008; Li et al., 2008)</td>
</tr>
<tr>
<td>PD0325901</td>
<td>MEK inhibitor</td>
<td>facilitates conversion of mEpiSCs and hESCs to naïve pluripotent state;</td>
<td>(Hanna et al., 2010; Zhou et al., 2010)</td>
</tr>
<tr>
<td>PD0325901</td>
<td>MEK inhibitor</td>
<td>facilitates generation and maintenance of mESC-like rat or human iPSCs;</td>
<td>(Li et al., 2009a)</td>
</tr>
<tr>
<td>PD0325901</td>
<td>MEK inhibitor</td>
<td>facilitates rapid and efficient generation of fully reprogrammed hiPSCs;</td>
<td>(Lin et al., 2009)</td>
</tr>
<tr>
<td>PD0325901</td>
<td>MEK inhibitor</td>
<td>enables Oct4-mediated reprogramming when combined with A-83-01/NaB/PS48</td>
<td>(Zhu et al., 2010)</td>
</tr>
<tr>
<td>CHIR99021</td>
<td>GSK3 inhibitor</td>
<td>Supports ESCs self-renewal; facilitates ESCs derivation from refractory stains or species</td>
<td>(Ying et al., 2008; Tsutsui et al. 2011)</td>
</tr>
<tr>
<td>CHIR99021</td>
<td>GSK3 inhibitor</td>
<td>captures and maintains lineage-specific stem cells, like pNSCs; facilitates the conversion of mEpiSCs and hESCs to naïve pluripotent state;</td>
<td>(Nichols et al., 2009; Buehr et al., 2008; Li et al., 2008)</td>
</tr>
<tr>
<td>CHIR99021</td>
<td>GSK3 inhibitor</td>
<td>enables Oct4- and Klf4-mediated reprogramming of MEFs or human primary keratinocytes with Parnate;</td>
<td>(Li et al., 2009a; Hanna et al., 2010; Zhou et al., 2010; Li et al., 2009b)</td>
</tr>
<tr>
<td>CHIR99021</td>
<td>GSK3 inhibitor</td>
<td>facilitates generation and maintenance of mESC-like rat or human iPSCs;</td>
<td>(Li et al., 2009a)</td>
</tr>
<tr>
<td>CHIR99021</td>
<td>GSK3 inhibitor</td>
<td>facilitates the neural conversion of human fibroblasts mediated by Ascl1 and Ngn2</td>
<td>(Ladewig et al., 2012)</td>
</tr>
<tr>
<td><strong>Compound name</strong></td>
<td><strong>Identity</strong></td>
<td><strong>Function</strong></td>
<td><strong>References</strong></td>
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<tr>
<td>6-bromoindirubin-3'-oxime (BIO)</td>
<td>GSK3 inhibitor</td>
<td>Promotes self-renewal of ESCs and Isl+ cardiovascular progenitors</td>
<td>(Sato et al., 2004; Qyang et al., 2007)</td>
</tr>
<tr>
<td>Kenpaullone</td>
<td>GSK3 and CDK inhibitor</td>
<td>Replaces Klf4 in MEF reprogramming</td>
<td>(Lyssiotis et al., 2009)</td>
</tr>
<tr>
<td>PD173074</td>
<td>FGF receptor inhibitor</td>
<td>Supports mESC self-renewal;</td>
<td>(Buehr et al., 2008)</td>
</tr>
<tr>
<td>PD173074</td>
<td>FGF receptor inhibitor</td>
<td>facilitates the conversion of mEpiSCs to naïve pluripotent state</td>
<td>(Zhou et al., 2010)</td>
</tr>
<tr>
<td>SU5402</td>
<td>FGF receptor inhibitor</td>
<td>Supports mESC self-renewal</td>
<td>(Buehr et al., 2008)</td>
</tr>
<tr>
<td>A-83-01</td>
<td>ALK4, ALK5, ALK7 inhibitor</td>
<td>Facilitates the conversion of mEpiSCs to naïve pluripotent state;</td>
<td>(Zhou et al., 2010)</td>
</tr>
<tr>
<td>A-83-01</td>
<td>ALK4, ALK5, ALK7 inhibitor</td>
<td>enables generation and long-term maintenance of mESC-like human iPSCs;</td>
<td>(Li et al., 2009a)</td>
</tr>
<tr>
<td>A-83-01</td>
<td>ALK4, ALK5, ALK7 inhibitor</td>
<td>enables Oct4-mediated reprogramming when combined with PD0325901/NaB/PS48</td>
<td>(Zhu et al., 2010)</td>
</tr>
<tr>
<td>SB431542</td>
<td>ALK4, ALK5, ALK7 inhibitor</td>
<td>Captures and maintains pNSCs when combined with CHIR99021;</td>
<td>(Li et al., 2011)</td>
</tr>
<tr>
<td>SB431542</td>
<td>ALK4, ALK5, ALK7 inhibitor</td>
<td>facilitates rapid and efficient generation of fully reprogrammed human iPSCs;</td>
<td>(Lin et al., 2009)</td>
</tr>
<tr>
<td>SB431542</td>
<td>ALK4, ALK5, ALK7 inhibitor</td>
<td>Facilitates the neural conversion of human fibroblasts mediated by Ascl1 and Ngn2</td>
<td>(Ladewig et al., 2012)</td>
</tr>
<tr>
<td>E-616452</td>
<td>ALK4, ALK5 and ALK7 inhibitor</td>
<td>Replaces Sox2 in MEF reprogramming.</td>
<td>(Ichida et al., 2009; Maherali and Hochedlinger, 2009)</td>
</tr>
<tr>
<td>Compound E</td>
<td>γ-secretase inhibitor</td>
<td>Accelerates the generation of pNSCs</td>
<td>(Li et al., 2011)</td>
</tr>
<tr>
<td>Compound name</td>
<td>Identity</td>
<td>Function</td>
<td>References</td>
</tr>
<tr>
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</tr>
<tr>
<td>JAK Inhibitor I</td>
<td>JAK inhibitor</td>
<td>Inhibits the generation of iPSCs in iPSC-TF-based transdifferentiation</td>
<td>(Efe et al., 2011; Kim et al., 2011a)</td>
</tr>
<tr>
<td>Pluripotin (SC1)</td>
<td>RasGAP and ERK inhibitor</td>
<td>Maintains mESC self-renewal</td>
<td>(Chen et al., 2006)</td>
</tr>
<tr>
<td>Y-27632</td>
<td>ROCK inhibitor</td>
<td>Improves survival of hESCs upon dissociation</td>
<td>(Chen et al., 2010; Ohgushi et al., 2010; Xu et al., 2010)</td>
</tr>
<tr>
<td>Thiazovavin (Tzv)</td>
<td>ROCK inhibitor</td>
<td>Improves survival of hESCs upon dissociation; facilitates rapid and efficient generation of fully reprogrammed hiPSCs</td>
<td>(Xu et al., 2010)</td>
</tr>
<tr>
<td>Thiazovavin (Tzv)</td>
<td>ROCK inhibitor</td>
<td>Improves survival of hESCs upon dissociation; facilitates rapid and efficient generation of fully reprogrammed hiPSCs</td>
<td>(Lin et al., 2009)</td>
</tr>
<tr>
<td>StemRegenin1</td>
<td>AhR antagonist</td>
<td>Enables ex vivo expansion of CD34+ HSCs ex vivo</td>
<td>(Boitano et al., 2010)</td>
</tr>
<tr>
<td>PS48</td>
<td>PDK1 activator</td>
<td>Enables OCT4-mediated reprogramming with A-83-01, NaB and PD0325901</td>
<td>(Zhu et al., 2010)</td>
</tr>
<tr>
<td>BayK8644</td>
<td>L-type Ca(^{2+}) channel agonist</td>
<td>Promotes MEF reprogramming mediated by Oct4 and Klf4 when combined with BIX-01294</td>
<td>(Shi et al., 2008a)</td>
</tr>
<tr>
<td>Forskolin</td>
<td>PKA agonist</td>
<td>Induces Klf4 and Klf2 expression to facilitate hESCs conversion into a naïve pluripotent state</td>
<td>(Hanna et al., 2010)</td>
</tr>
</tbody>
</table>
chemical inhibitors can remodel the epigenetic landscape of the somatic cell type to be more like that of ESC by removing the repressive epigenetic marks and relaxing chromatin structure to allow the reprogramming factors easier access to target genes.

DNA Methyltransferase Inhibitors

Methylated CpG sites in gene promoter regions are easily recognized by specific methyl CpG binding proteins, which act as adapters between methylated DNA and chromatin modifying factors. These proteins can recruit co-repressors, such as histone deacetylases (HDAC), methyltransferases, and chromatin remodeling factors, creating a protein complex that regulates gene expression. If the promoter region is methylated, the corresponding gene's expression is suppressed due to its' inability to recognize transcription factors (Gnyszka et al., 2013).

5-azacytidine (AZA) is the most commonly used DNA methyltransferase (DNMT) inhibitors. Once incorporated into the genome, 5-AZA disrupts the interaction between DNA and DNMTs through nitrogen, instead of carbon, in the 5-position of the modified pyrimidine (Gnyszka et al., 2013). The enzyme remains covalently bound to DNA and its DNMT, thus blocking its function. Additionally, the covalent protein adduction also compromises the functionality of DNA and triggers DNA damage signaling, resulting in the degradation of trapped DNMTs. As a result, further methylation of cytosine residues is inhibited, causing the passive loss of cytosine methylation in daughter cells (Stresemann and Lyko, 2008).

As previously mentioned, the vast majority of cells subjected to direct reprogramming conditions are trapped in an intermediate state that is characterized by
the downregulation of somatic genes, maintenance of viral transgene expression, incomplete reactivation of pluripotent genes, inability to form chimeras, and persistent DNA hypermethylation. Mikkelsen et al., (2008) demonstrated that these partially reprogrammed cells can be coaxed to undergo complete reprogramming by treatment with 5-AZA. Furthermore, treatment with 5-AZA was also shown to improve the number of ESC-like colonies by 4-fold (Mikkelsen et al., 2008).

Histone Deacetylase Inhibitors

Acetylation plays an important role in nucleosome assembly and chromatin folding. Acetylation favors an open chromatin structure by interfering with the interactions between nucleosomes and releasing the histone tails from the linker DNA. Chromatin regions that are marked by lysine acetylation catalyzed by Histone Acetyltransferase (HATs) are generally actively transcribed, whereas regions that are bound by Histone Deacetylases (HDACs) bear deacetylated lysines and are inactive (Shahbazian and Grunstein, 2007; Kretsovali et al., 2012).

HDAC inhibitors (HDACis) are small molecules that inhibit the activities of HDACs, thus playing a major role in epigenetic regulation. Consequently, HDACis have been widely utilized in nuclear reprogramming. Trichostatin A (TSA) and valproic acid (VPA) are among the most commonly used HDACis, and their role as an effective facilitator of nuclear reprogramming has been well documented. Huangfu and colleagues (2008a) demonstrated the ability to generate iPSC in the absence of c-myc by substituting it for VPA. Using the pluripotency factors Oct-4, Sox-2, and Klf-4 and VPA, they were not only able to reprogram primary human fibroblast cells into iPSC, but
they were also able to increase the number of iPSC colonies by 50-fold. In another study by Huangfu and colleagues (2008b), it was shown that the combination of Oct-4, Sox-2, and VPA was sufficient to reprogram somatic cells at an efficiency similar to that of three factor reprogramming. It should be noted that other combinations of two factors did not yield any iPSC, even in the presence of VPA, suggesting that Oct-4 and Sox-2 are indispensable in reprogramming human fibroblasts in the context of VPA treatment. That being said, mouse embryonic fibroblasts (MEFs) treated only with VPA exhibited an upregulation of ESC-specific genes, while MEF-specific genes were downregulated. This suggests that VPA may support a predisposition towards an ESC-like state. Furthermore, the genome-wide acetylation induced by VPA and other HDACis could allow MEFs to adopt a relaxed chromatin structure that facilitates the binding of ectopically expressed transcription factors or downstream secondary factors (Feng et al., 2009).

Other Small Molecules

Histone lysine methylation plays a key role in the organization of chromatin domains and the regulation of gene expression; however, aberrant expression of histone lysine methyltransferases (HMTases) like G9a has been linked to tumor development. G9a is a H3K9 methyltransferase, which is localized exclusively in euchromatic regions. It functions to repress gene activity by inducing local H3K9me2 and H3k9me3 at target promoters (Kubicek et al., 2007). In addition to inducing di- and tri-methylation of H3K9, G9a methylates K373 of p53, a tumor suppressor. Overexpression of G9a results in the inactivation of p53, which is implicated in over 50% of cancers (Huang et al., 2010). Studies have shown that knockout of G9a inhibits
cancer growth (McGarvey et al., 2006; Kondo et al., 2008), leading researchers to explore G9a inhibition as a potential approach for cancer treatment.

BIX-01294 has been identified as a small-molecule inhibitor of G9a. BIX-01294 does not compete with the cofactor S-adenosyl-methionine, and is highly selective for G9a. Importantly, BIX-01294 is biologically active in reducing H3K9me2 levels at several G9a target genes, thereby allowing for the transient reversal of this repressive mark in vitro (Kubicek et al., 2007). Among the many genes G9a HTMase regulates is the pluripotency gene, Oct-4. The quest to generate iPSC efficiently and without viral genome integration lead Shi et al (2008a) in search of a chemical cocktail that would allow reprogramming of somatic cells in chemically defined conditions. In this study, neural progenitor cells (NPC), which endogenously express Sox-2 (Blelloch et al., 2006), were transduced with only Oct-4 and Klf-4 and were successfully reprogrammed to iPSC. They found reprogramming to be greatly enhanced by the presence of BIX-01294. Furthermore, BIX-01294 was shown to enable reprogramming of NPC transduced with c-Myc, Klf-4, and Sox-2, in the absence of Oct-4 ectopic expression. In this particular system, BIX-01294 seemed to compensate for the lack of Oct-4 overexpression (Shi et al., 2008a). In a follow-up study by Shi and colleagues, they aimed to assess if a similar strategy could be used to find small molecules that can replace viral transduction to obtain iPSC from a general cell line, mouse embryonic fibroblasts (MEF), in which none of the transcription factors deemed essential for reprogramming, Oct-4, Sox-2, and Klf-4, are expressed. They found that a combination of BIX-01294 and Bayk8644, a L-channel calcium agonist, was effective at enabling the generation of iPSC from MEF transduced with only Oct-4 and Klf-4 (Shi et al., 2008b).
Their interest in Bayk8644 stems from the fact that it exerts its effects upstream in cell signaling pathways and does not directly cause epigenetic modifications; therefore, it can likely be exploited to induce reprogramming in a more specific manner than molecules acting directly at the epigenetic level causing DNA or histone modification (Shi et al., 2008b). Activation of L-type calcium channels by different agonists, including Bayk8644, has been shown to induce intracellular signaling through CREB activation, sacoplasmic reticulum calcium release, and change in cAMP activity. Furthermore, it has been suggested that calcium may play a role in the control of mESC proliferation (Heo et al., 2006). However, Shi et al (2008b) observed no change in proliferation when mESC were treated with Bayk8644 alone or in combination with BIX-01294. Treatment of MEF with Bayk8644 alone or in combination with BIX-01294 did not induce Sox-2 expression either, making it rather interesting that Bayk8644 improves reprogramming efficiency. While further work is needed to dissect the exact mechanism by which Bayk8644 improves the reprogramming process, it is interesting to find that a small molecule with activity in signaling pathways that have not been previously linked to reprogramming can significantly enhance its efficiency (Shi et al., 2008b). To date, it is the only small molecule of its type to show an effect of reprogramming, as most of the other small molecules identified appear to directly modify the epigenetic status of the cell: BIX-01294 (Shi et al., 2008a), VPA (Huangfu et al., 2008), and 5-AZA (Mikkelsen et al., 2008). Because Bayk8644 does not reprogram on its own but requires the presence of BIX-01294 to exert its effects, this suggests that cells that are already undergoing a form of reprogramming, may be more susceptible to its effect, making it
possible to reprogram the target cell in a more specific manner, without impacting healthy cells systemically, as direct epigenetic modifier might (Shi et al., 2008b).

Small molecules are also employed to target specific signaling pathways that coincide with the pluripotent state. The role Leukemia Inhibitory Factor (LIF) and Bone Morphological Protein (BMP) play in the self-renewal of murine ESC has been well established (Niwa et al., 1998); therefore, it was surprising to discover that mESCs could sustain their identity in the absence of LIF and BMP. Chen and colleagues (2006) identified a synthetic small molecule known as Pluripotin, which enabled mESC to maintain long-term self-renewal under feeder-, serum-, and LIF-free conditions. Pluripotin inhibits two endogenous differentiation-inducing proteins, Ras GTPase-activating protein (RasGAP) and ERK1 (also known as MAPK3), thus allowing pluripotency to be sustained without the use of exogenous factors to activate pluripotency-associated pathways. The identification of Pluripotin revealed a fundamental strategy for maintaining stem cell self-renewal through the inhibition of endogenous differentiation mechanisms, and explained how combining the activation of differentiation-inducing pathways with the modulation of other pathways can sustain self-renewal by effectively balancing out the differentiation activity of stem cells (Zhang et al., 2012). Similarly, PD0325901, a mitogen activated protein kinase/ERK kinase (MEK) inhibitor, and CHIR99021, a glycogen synthase kinase-3 (GSK3) inhibitor, have also been shown to facilitate the maintenance of mESC without the need for feeder cells or exogenous cytokines (Buehr et al., 2008). Inhibition of both the MEK and GSK3 pathways eliminates the need for LIF and BMP in mESCs. Stimulation of the ERK signaling pathway triggers the transition from pluripotency to lineage-commitment
 Increased understanding of signaling pathways and their role in cellular identity has greatly advanced iPSC technology. Although the strategy developed by Takahashi and Yamanaka for generating iPSC has enormous therapeutic potential, it is not an infallible technique, and, despite substantial progress in recent years, a number of challenges remain. Reprogramming remains a largely inefficient and non-specific process, with efficiencies of transduced cells becoming fully reprogrammed iPSCs lower than 0.01% (Takahashi et al., 2007; Yu et al., 2007; Hasegawa et al., 2010). The majority of transduced cells become trapped in a pre-iPSC state that is characterized by the downregulation of somatic cell marker genes, incomplete reactivation of pluripotency genes, maintenance of viral expression, and the inability to form chimeras. To achieve complete reprogramming, DNA methylation, histone modification, and chromatin structure need to mimic that of the embryonic environment. The introduction of key transcription factors (Oct-4, Sox-2, Klf-4, c-myc) initiate a number of complex processes that cooperatively function to reboot the epigenetic state of the somatic cell type. C-myc is believed to loosen the tight chromatin structure characteristic of somatic cells, which allows Oct-4 and Sox-2 to co-bind their target genes to launch the pluripotent network (Takahashi and Yamanaka, 2006; Wernig et al., 2008). Among these targets are a group of genes that encode for epigenetic factors that participate in the maintenance of self-renewal and pluripotency (Loh et al., 2006). Several studies have shown that small molecules can improve reprogramming when combined with
conventional reprogramming factors (Huangfu et al., 2008; Mikkelsen et al., 2008; Shi et al., 2008b). The relief of repression on key pluripotency genes such as Oct-4 and Nanog may allow an earlier induction of self-renewal and pluripotency (Feng et al., 2009). Similarly, Silva and colleagues (2008) demonstrated that partially reprogrammed cells can be coaxed out of this state and transition to fully reprogrammed iPSCs by treatment with the chemicals PD0325901 and CHIR99021 to inhibit MEK and GSK3, respectively. These chemical inhibitors could potentially induce a global permissive epigenetic landscape similar to ESCs by inducing epigenetic changes that promote the active transcriptional state and facilitating the erasure of repressive epigenetic features. The combination of events may provide exogenous reprogramming factors and secondarily-induced transcription factors greater access to downstream target genes. Therefore, genetic factors and small molecules may synergistically modify epigenetic features to activate the pluripotent transcriptional network to enhance reprogramming (Feng et al., 2009).

**Conclusion**

In 2006, Shinya Yamanaka made a groundbreaking discovery that would ultimately win him the Nobel Prize in Physiology and Medicine just 6 years later. The discovery of iPSC represents the culmination of over 60 years of research, which began by asking the simple question: “is cell fate irreversibly fixed as cells become more specialized?” The progression from nuclear transfer in frogs to the generation of patient-specific iPSC echoes scientists’ expanding knowledge of the molecular machinery that regulate cell fate. Collectively, these studies prove that a differentiated cell can be reverted back to earlier state. The resetting of the somatic cell nucleus to an
embryonic state involves several complex mechanisms, and, despite an increased understanding of reprogramming mechanisms, much is still to be learned before its full potential can be harnessed. Nevertheless, research in the field of iPSC is steadfast and progress is continuously being made.

Studies have revealed that epigenetics play a crucial role in nuclear reprogramming. Resistance to reprogramming is largely attributed to the phenomenon of epigenetic memory that cells retain throughout numerous cell divisions. As cells become more differentiated, they acquire epigenetic marks that make their nuclei increasingly resistant to reprogramming (Pasque et al., 2011). However, modulations of the epigenetic processes that accompany nuclear reprogramming may facilitate the conversion to an epigenetic landscape that is more permissive to reprogramming. Various tools have been employed to help cells overcome this reprogramming roadblock, including the use of small molecules. Several chemical compounds that control epigenetic enzymes, such as HDAC, HMT, DNMT, and histone demethylase (HDM) have been shown to improve reprogramming efficiency, or even replace the need to use certain transcription factors (Zhang et al., 2012). This is especially promising for the clinical application of iPSC as there are serious concerns regarding the safety of current genetic approaches to nuclear reprogramming, as well as traditional culture systems that are used to maintain iPSC. Small molecules provide an attractive approach to addressing these challenges, as they offer a number of compelling advantages. First, the biological effects of small molecules are typically rapid, reversible and dose-dependent, allowing precise control over specific outcomes by fine-tuning their concentrations and combinations. Second, the structural diversity
that can be provided by synthetic chemistry allows the functional optimization of small molecules. Third, compared with genetic interventions, the relative ease of the handling and administration of small molecules make them more practical for in vitro and in vivo applications, and for further therapeutic development (Zhang et al., 2012).
CHAPTER III
EFFECT OF SMALL MOLECULE TREATMENT ON EXPRESSION OF PLURIPOTENCY GENES IN BOVINE FETAL FIBROBLAST CELLS

Introduction

Successful reprogramming of somatic cells towards pluripotency requires the epigenetic marks characteristic of the differentiated cell type first be erased in order to inactivate the somatic cell program and activate the embryonic program. However, the majority of cells subjected to reprogramming conditions become trapped in a partially reprogrammed state that is characterized by the downregulation of somatic cell marker genes, incomplete reactivation of pluripotency genes, maintenance of viral expression, and the inability to form chimeras. To achieve complete reprogramming, DNA methylation, histone modification, and chromatin structure need to mimic that of the embryonic environment. Several small molecules, affecting specific signaling pathways and/or chromatin modifications, have been shown to improve both the kinetics and efficiency of reprogramming (summarized in Table 2.1). In pharmacology, a small molecule is defined as low molecular weight (<900 Daltons) organic bioactive compounds that may help regulate a biological process. These chemical modifiers aid in overcoming the “roadblocks” encountered during the reprogramming process by inducing the necessary epigenetic modifications needed to silence the somatic cell genome and completely reactivate the ESC genome. Chemical treatment of cells prior to reprogramming can remodel the epigenetic landscape of the somatic cell type to be more like that of ESC by removing the repressive epigenetic marks and relaxing chromatin structure to allow the reprogramming factors easier access to target genes.
In the decade since Takahashi and Yamanaka's (2006) groundbreaking discovery, much effort has been made to better understand the molecular circuitry permitting the generation of iPSC in the hopes of improving this reprogramming method. Reprogramming by defined factors is an ineffective process, with efficiencies of transduced cells becoming fully reprogrammed iPSC lower than 0.01% (Takahashi et al., 2007; Yu et al., 2007; Hasegawa et al., 2010). Furthermore, safety concerns regarding the use of exogenous genes and the method of delivering these reprogramming factors, as well as technical and logistical challenges have hindered clinical applications of iPSC. Scientists tackled these issues with steadfastness, and a number of genetic factors, chemical inhibitors, and signaling molecules have been shown to either promote or enhance reprogramming. Concerns over genome modification through exogenous sequences were largely resolved by the introduction of new delivery methods, which included the use of episomal plasmids (Yu et al., 2009) or excisable expression systems (Soldner et al., 2009), recombinant cell-penetrating reprogramming proteins (Kim et al., 2009; Zhou et al., 2010) and reprogramming mRNAs (Warren et al., 2010; Yakubov et al., 2010) or microRNAs (Anokye-Danso et al., 2011; Miyoshi et al., 2011). Although, genetic methods have been widely used to address these issues, a chemical approach offers many advantages. First, the biological effects of small molecules are typically rapid, reversible and dose-dependent, allowing precise control over specific outcomes by fine-tuning their concentrations and combinations. Genetic approaches, on the other hand, involve permanent genome modifications with associated problems of tumorigenicity and other irreversible, unintended consequences. Second, the structural diversity that can be provided by
synthetic chemistry allows the functional optimization of small molecules. Whereas biological reagents are best suited for targeting extracellular components, small molecules are cell-permeable and, therefore, have the potential to target every class of macromolecule in the cell. Third, compared with genetic interventions, the relative ease of the handling and administration of small molecules make them more practical for in vitro and in vivo applications, and for further therapeutic development (Zhang et al., 2012). Because of the advantages, interest in utilizing small molecules in stem cell biology and regenerative medicine has significantly heightened. Table 3.1 summarizes the growing number of small molecules that have been identified to maintain self-renewal potential of stem cells, to induce lineage differentiation, and to facilitate reprogramming either by increasing efficiency or by replacing genetic reprogramming factors. Identification of novel small molecules compounds that affect cell fate and increased understanding of the nuclear reprogramming process lead Hou et al. (2013) to report the first-ever all chemical generation of mouse iPSC from MEF cells. The Chemically-induced Pluripotent Stem Cells (CiPSCs) were induced through a very complicated procedure with 3-step compound treatment shown in Figure 3.1. After a year of screening 10,000 compounds, they concluded that a cocktail of seven small molecules was capable of cell fate reprogramming. The seven small molecule cocktail, known as “VC6TFZ”, is comprised of VPA, CHIR99021, 616452, Tranylcypromine, Forskolin (FSK), 2-methyl-5-hydroxytryptamine (2-Me-5HT), and D4476. Hou et al. (2013) identified this potent cocktail by first generating iPSC using the Oct-4 gene in combination with CHIR99021, 616452, and Tranylcypromine.
Next, they screened different chemical substitutes for Oct-4 using a Oct-4 promoter-driven GFP expression system. After screening 10,000 compounds, they concluded FSK, 2-Me-5HT, and D4476 could substitute for Oct-4. VC6TF treatment yielded some GFP-positive cell clusters, however neither Oct-4 nor Nanog were detectable in these cells, indicating incomplete reprogramming. They identified one final chemical, 3-deazaneplanocin (DZNep), to be critical later in the reprogramming process for activating endogenous Oct-4. They designed CiPSC protocol including 3 steps as follows: (a) MEF cells were cultured in mESC medium containing VC6FT for 16–20 days; (b) cells were cultured in the medium with VC6FTZ for 12–20 days; (c) cells were cultured in mESC medium containing 2i (PD0325901 and CHIR99021) for 1 week (Hou et al., 2013).

Figure 3.1 Schematic diagram illustrating direct iPSC reprogramming from MEF using only small molecules. Hou et al.,(2013) reported that CiPSC generation from MEF was carried out in three steps of 16-20 days in VC6TF treatment, and then 12-20 days in VC6TFZ followed by the application of two MEK and GSK3-β inhibitors, known as “2i”, to finalize chemical reprogramming. Using a doxycycline (DOX)-inducible GFP-Oct-4 expression screening system, ectopic GFP-Oct-4 expression was induced during the first round, followed by DOX withdrawal and small molecule treatment.
Chemical-only reprogramming is a long, multi-step, sequence-dependent process characterized by a unique intermediate state. The cells at this intermediate state have a strong resemblance to embryo-derived extraembryonic endoderm (eXEN) cells (Zhou et al., 2010). eXEN cells are derived from primitive endoderm cells in late blastocyst stage embryos and continue to propagate in culture while retaining their ability to contribute to extraembryonic cell lineage after injection into blastocysts (Kunath et al., 2007). The intermediate cells of chemical reprogramming, designated as chemically-induced XEN (CeXEN) cells, express Gata4, Gata6, Sox17, Sox7, and Sall4, and also possess the ability to contribute to the extraembryonic cell lineages in chimeric embryos. Moreover, both eXEN and CeXEN cells are competent to be reprogrammed to the pluripotent state by the second step of chemical reprogramming cocktail, indicating their stringent similarity in their cell biological characteristics. It has been shown that the knockdown of the endoderm-associated TFs such as Gata6 compromises the first step of chemical reprogramming, whereas its overexpression with additional TF Sall4 replaces some components of the first step chemical reprogramming cocktail, confirming the functional importance of the XEN-like state as an intermediate (Zhou et al., 2010).

XEN-like state is an intermediate of the chemical reprogramming, but not of TF-mediated reprogramming. Some small molecules have demonstrated the ability to replace individual Yamanaka factors; however, no single combination of small molecules is capable of replacing them all. Intrigued by this dubious discovery, Shu et al. (2013) performed many studies to discern the chemical-based reprogramming mechanism. They proposed a new “Seesaw” model in which the pluripotent state is a
precarious balancing equilibrium that results from continuous mutual competition between rival lineage specification forces. Pluripotency-inducing reprogramming factors have been identified primarily from pluripotency-associated factors in ESC; however, pluripotency can be induced with lineage specifiers that suppress ESC identity using pluripotency rivals, most of which are not enriched in ESCs. To that end, Shu et al. (2013) reported that Oct-4 and Sox-2 can be replaced by lineage specifiers that are involved in mesendodermal (ME) and ectodermal (ECT) specification, respectively. Oct-4 and its substitutes attenuated the elevated expression of a group of ECT genes, whereas Sox-2 and its substitutes curtailed a group of ME genes during reprogramming. Moreover, they found that the two counteracting lineage specifiers could synergistically induce pluripotency in the absence of both Oct-4 and Sox-2 (Shu et al., 2013). Based on these findings, they concluded that chemical-based reprogramming is mediated by the counteracting lineage specifier compounds, VC6FTZ.

Insight into the mechanisms that govern nuclear reprogramming has propelled the advancement and improvement of reprogramming strategies. Therefore, we sought to assess the genetic and epigenetic effects of pre-treatment with small molecules on the core pluripotency-determining transcription factors, Oct-4, Nanog, and Sox-2. In this first study, we measured the effect of small molecule treatment on Oct-4, Nanog, and Sox-2 transcript expression in bovine fetal fibroblast (BFF) cells.
Materials and Methods

Experiment 1 Experimental Design

In the present study, we assessed the effect of pre-treatment with small molecule inhibitors on the expression of Oct-4, Nanog, and Sox-2 in BFF cells. BFF cells were allowed to expand in culture before being split evenly into two groups. Cells in the control group were cultured in complete culture medium (DMEM + 10% FBS + 1% P/S), whereas cells in the treatment group were cultured in complete culture medium supplemented with 0.5mM PD0325901, 3mM CHIR99021, and 1.8mM NuP0178 for the duration of 7 days. On day 7, mRNA was isolated from control and treatment cell cultures for gene expression analysis. Quantitative (qRT-PCR) was performed to measure transcript levels for Oct4, Nanog, and Sox-2 in control and treatment BFF cells.

Experiment 2 Experimental Design

To explore what, if any, effects a longer period of treatment with PD325901, CHIR99021, and NuP0178 has on Oct-4, Nanog, and Sox-2 expression in BFF cells, we extended the culture period to 14 days. Using the same design and procedures as Experiment 1, control and treatment cells were cultured in their respective culture system for a total of 14 days. On day 14, mRNA was isolated from both control and treatment cell cultures for gene expression analysis. Quantitative (qRT-PCR) was performed to measure transcript levels for Oct4, Sox2, Nanog in control and treatment BFF cells.
Establishment of Cell Lines and Maintenance

Target cell lines were obtained from previously isolated primary cultures. Primary cultures of fibroblast cells were established from bovine fetuses approximately 50 days old according to protocol (Giraldo et al., 2009; Coley, 2010). BFF cells were cultured in complete culture medium composed of Dulbecco’s Modified Eagle Medium (DMEM) with high glucose, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (P/S). Once cells reached 80% confluency, cultures were passaged by releasing cells with trypsin (0.25%). BFF cells were then resuspended in DMEM supplemented with 10% bovine calf serum (CS) and 10% dimethyl sulfoxide (DMSO) for cryopreservation. Cryovials containing approximately 1,000,000 BFF cells suspended in solution were cooled at 1°C/min until reaching -80°C before storage in liquid nitrogen.

BFF cells were thawed as needed at room temperature for 30 sec, followed by submersion in a 37°C water bath. Cells were then washed in complete culture medium and seeded at a density of 0.7 x 10^6 into 25-cm² flasks and cultured under 5% CO₂ and 90% humidity at 37°C. Because the experimental procedure required a high volume of cells, BFF cells were expanded through repeated passaging until a sufficient number of cells were obtained prior to each experiment.

Treatment with Small Molecules

Chemical treatment consisted of 3 small molecule inhibitors (3i): PD0325901 (Stemgent, Lexington, MA, Cat.no. 04-0006), a mitogen activated protein kinase/ERK kinase (MEK) inhibitor; CHIR99021 (Stemgent, Lexington, MA, Cat.no. 04-0004), a
glycogen synthase kinase-3 (GSK3) inhibitor; and NuP0178 (NuPotential, Baton Rouge, LA), a G9a histone methyltransferase inhibitor. A 10mM stock solution of each inhibitor was prepared by dissolving the crystalline solid in DMSO. 3i+ media was prepared by diluting each stock into complete culture medium (DMEM, 10% FBS, 1% P/S) to a concentration of 0.5µM PD0325901; 3µM CHIR99021; 1.8µM NuP0178. The concentrations of CHIR99021 and PD0325901 utilized were consistent with previous studies (Yu et al., 2011) and NuP0148 concentration was estimated due to its recent development and concentration evaluation experiments carried out by NuPotential, LLC.

Once BFF cells were expanded to an adequate quantity, trypsin (0.25% EDTA) was added to confluent cultures of BFF cells to disaggregate cells adherent to the flask, which were then counted using a hemocytometer. Cells were then divided evenly into treatment and control groups, approximately 2.5 x 10^6 cells per group. Control cells were resuspended in complete culture medium and split evenly between three 25-cm^2 flasks (approximately 0.7 x 10^6 per flask). Similarly, treatment cells were resuspended in 3i+ media and split evenly between three 25-cm^2 flasks (approximately 0.7 x 10^6 per flask). All flasks were then placed in 37°C incubator with 5% CO_2 and 90% humidity. Media was changed every 1-2 days, and cells were passaged as needed.

Isolation of mRNA

On days 7 and 14, both treatment and control cells were harvested by trypsinization and washed in 1 ml of Dulbecco’s Phosphate Buffered Solution (DPBS) with Ca^{+2} and Mg^{+2}. Cells were pelleted by centrifugation, and the cell pellet was placed in -80°C freezer for later use. mRNA was isolated from both treatment and control cells
using Dynabeads® mRNA Direct™ Kit (Invitrogen Dynal As, Oslo, Norway, Cat.no. 610.11/610.12) as described previously by Wrenzycli et al (2001). Frozen cell pellets were removed from -80°C freezer, and 1.25 ml of lysis/binding buffer (100 mM Tris-HCl (pH 7.5), 500 mM LiCl, 10 mM EDTA (pH 8), 1% lithium dodecylsulfate (LiDS), and 5 mM dithiothreitol (DTT)) was immediately added to the frozen cell pellet. Cell suspension was repeatedly passaged through a pipette tip to facilitate lysis; however because samples contained more than 5 x 10^5 cells, a DNA-shear step was also performed. DNA was sheared by forcing the lysate through using a 21 gauge needle five times to obtain complete cell lysis. Next, the sample lysate (1.25 ml) was added to 250 µl of pre-washed Dynabeads® Oligo(dT)_{25} and resuspended completely. The bead-lysate mixture was incubated for 5 min on a rotating mixer to allow the polyA tail of the mRNA to hybridize to the oligo (dT)_{25} on the beads. The beads were separated from the mix using a Dynal MPC-E-1 magnetic separator. The beads/mRNA complex was then washed twice in 1 ml of Washing Buffer A (10 mM Tris-HCl (pH 7.5), 150 mM LiCl, 1 mM EDTA, and 0.1% LiDS), followed by two washes with 1 ml of Washing Buffer B (10 mM Tris-HCl (pH 7.5), 150 mM LiCl, and 1 mM EDTA). mRNA was eluted from the beads by adding 25 µl of 10 mM Tris-HCl (pH 7.5) and incubating for 2 min at 75°C. Immediately after incubation, tubes were placed in the Dynal MPC-E-1 magnetic separator to separate the beads from the mRNA. The supernatant containing the mRNA was transferred to a new RNase-free tube and placed on ice for succeeding use in reverse transcription.
Reverse Transcription

The freshly-isolated mRNA was reverse transcribed into cDNA in a total volume of 25 µl using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA, Cat.no. 1708890). Each 25 µl iScript RT Reaction mix consisted of 12.5 µl of sample mRNA, 1 µl of reverse transcriptase, 4 µl of 5X iScript reaction mix, and 7 µl of nuclease-free H$_2$O. The reaction was conducted at 25°C for 5 min, 42°C for 30 min, a denaturation step of 85°C for 5 min, and a final holding temperature of 4°C.

Quantitative Real-Time PCR

Our laboratory has previously analyzed BFF cells for Oct-4, Nanog, and Sox-2 expression, thus we have validated the specificity of primers for PAP, Oct-4, Nanog, and Sox-2 (Coley, 2010). All primers were designed from bovine gene sequences using the Beacon Designer 4.0 (PREMIER Biosoft International) (Table 3.2), and were diluted to 10 mM concentration. cDNA was amplified using SsoFast™ EvaGreen supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The total 20 µl real time PCR mix consisted of 2 µl of cDNA, 10 µl of SsoFast™ EvaGreen Supermix, 6 µl of nuclease-free water, and 1 µl of forward and reverse primer pairs (10 mM) for each gene. Within each qRT-PCR plate setup, reactions for the reference gene and each gene of interested were performed using the calibrator cDNA, the sample cDNA, and a no template negative control. Oct-4, Nanog, and Sox-2 expression levels in all samples was quantified using the Bio-Rad CFX Connect™ Real-Time PCR Detection System. The PCR program used for the amplification of all genes consisted of a denaturing cycle of 30 sec at 95°C; 40 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 4°C.

Data was quantified using the method for relative quantification in qRT-PCR described by Pfaffl (2001). Values are reported as relative transcription or the n-fold difference relative to a calibrator. A mixture of cDNA from BFF cells at multiple passages was used as a calibrator for all of the target genes. PAP was used as the internal reference gene. The threshold cycle value of the reference gene was used to normalize the target gene signals in each sample. The amount of target transcripts relative to the calibrator was calculated using the following equation: n-fold difference = Efficiency Target Gene$^{ΔCTT}$/ Efficiency Reference Gene$^{ΔCTR}$, where an efficiency value of two was assumed. The $ΔC_T$ (for the target gene) value was calculated by subtracting the sample $C_T$ value of the target gene from the calibrator $C_T$ value of the

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Primers</th>
<th>Amplicon Length</th>
</tr>
</thead>
</table>
| Oct-4 | NM174580 | Sense GGTTCTCTTTTGAAAGGTGTTT
Antisense ACACCTCGGACCACGTCTTTTC | 223 |
| Nanog | DQ069776 | Forward AATTCCAGCAGCAAATCAC
Reverse CCCTCCCTCAATTGACAC | 215 |
| Sox-2 | NM001105463 | Sense AGGACTGAGAAGAAGGAAGAGG
Antisense AAGAAGAGGGCAAACTGGAATC | 164 |
| PAP | X63436 | Sense AAGCAACTCCATCAACTACTG
Antisense ACGGACTGGTCTTCTATAGC | 169 |
target gene. The ΔC<sub>T</sub>R (for the reference gene) value was calculated by subtracting the sample C<sub>T</sub> value of the reference gene PAP from the calibrator C<sub>T</sub> value of the reference gene. Therefore, all target abundance levels were expressed as n-fold differences relative to a calibrator and normalized to the reference gene in order to compensate for PCR variations between runs.

Statistical Analysis

In this experiment, a completely random design with repeated measures was employed to assess any effect treatment with 3i+ media had on Oct-4, Nanog, and Sox-2 gene expression in BFF cells over time. The PROC MIXED with repeated measures analysis with autoregression of order one covariance was used in SAS statistical software to determine any statistical differences in relative gene expression between treatment and control groups over time.

Results

The values corresponding to the relative expression of Oct-4, Nanog, and Sox-2 in untreated BFF cells and BFF treated with 3i+ media at day 7 and day 14 are reported in tables 3.3 and 3.4, respectively. Statistical analysis comparing Oct-4, Nanog, and Sox-2 expression levels in treatment and control groups revealed no significance difference in gene expression between groups at either day 7 or day 14. Likewise, time did not have a significant effect on Oct-4, Nanog, and Sox-2 transcript levels in BFF cells cultured in the presence of the small molecule inhibitors, CHIR99021, PD0325901, and NuP0178. Therefore, there was no significant effect of the treatment and time interaction.
**Table 3.2.** Oct-4, Nanog, and Sox-2 Relative Expression in Treatment and Control BFF Cells on Day 7¹

<table>
<thead>
<tr>
<th>Sample</th>
<th>Oct-4</th>
<th>Nanog</th>
<th>Sox-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEZ 2 P4 CON (1)</td>
<td>20311.37</td>
<td>8364.13</td>
<td>474.41</td>
</tr>
<tr>
<td>BEZ 2 P4 TRMT (1)</td>
<td>14.83</td>
<td>1.75</td>
<td>4.59</td>
</tr>
<tr>
<td>BEZ 3 P4 CON</td>
<td>0.2793</td>
<td>0.9931</td>
<td>0.0151</td>
</tr>
<tr>
<td>BEZ 3 P4 TRMT</td>
<td>1.68</td>
<td>0.1199</td>
<td>0.0100</td>
</tr>
<tr>
<td>BEZ 2 P4 CON (2)</td>
<td>76.64</td>
<td>21.26</td>
<td>9.71</td>
</tr>
<tr>
<td>BEZ 2 P3 TRMT (2)</td>
<td>4.41</td>
<td>0.3686</td>
<td>0.1550</td>
</tr>
<tr>
<td>BEX 4 P3 TRMT</td>
<td>235.57</td>
<td>11.88</td>
<td>8.28</td>
</tr>
<tr>
<td>BEX 1 P3 CON</td>
<td>1.56</td>
<td>0.6242</td>
<td>0.0813</td>
</tr>
<tr>
<td>BEX 3 P4 CON (2)</td>
<td>3.92</td>
<td>2.53</td>
<td>0.1708</td>
</tr>
</tbody>
</table>

¹The relative expression of Oct-4, Nanog, and Sox-2 at day 7 was measured in five BFF cell lines, designated as BEZ 2, BEZ 3, BEX 4, BEX 1, and BEX 3. BEZ 2 served as a biological replicate, denoted as BEZ 2 (1) and BEZ 2 (2).
Table 3.3. Oct-4, Nanog, and Sox-2 relative expression in treatment and control BFF cells on day 14.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Oct-4</th>
<th>Nanog</th>
<th>Sox-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEZ 2 P5 CON</td>
<td>51063.33</td>
<td>5404.70</td>
<td>14066.74</td>
</tr>
<tr>
<td>BEZ 2 P4 TRMT</td>
<td>209.39</td>
<td>51.63</td>
<td>30.70</td>
</tr>
<tr>
<td>BEZ 3 P5 CON</td>
<td>0.8409</td>
<td>0.9593</td>
<td>10.3388</td>
</tr>
<tr>
<td>BEZ 3 P5 TRMT (1)</td>
<td>1.23</td>
<td>0.6690</td>
<td>0.6690</td>
</tr>
<tr>
<td>BEZ 3 P5 TRMT (2)</td>
<td>7.11</td>
<td>8.51</td>
<td>4.41</td>
</tr>
<tr>
<td>BEX 4 P6 TRMT</td>
<td>9.32</td>
<td>2.16</td>
<td>0.5946</td>
</tr>
</tbody>
</table>

The relative expression of Oct-4, Nanog, and Sox-2 at day 14 was measured in three BFF cell lines, designated as BEZ 2, BEZ 3, and BEX 4. BEZ 3 served as a biological replicate, denoted as BEZ 3 (1) and BEZ 3 (2).

Discussion

The small molecule inhibitors, CHIR99021 and PD325901, have been widely used as tools to facilitate the generation and maintenance of iPSC (Li et al., 2009; Lin et al., 2009; Hanna et al., 2010; Zhou et al., 2010). In these cases, CHIR99021 and PD325901 aid in overcoming the “roadblocks” encountered during the reprogramming process by inducing the necessary epigenetic modifications needed to silence the somatic cell genome and completely reactivate the ESC genome. Interestingly, CHIR99021 and PD0325901 have been found capable of maintaining mESC in culture without the need of feeder cells or exogenous cytokines (Buehr et al., 2008). Inhibition of both the GSK3 and MEK pathways by CHIR99021 and PD0325901, respectively,
eliminates the need for LIF and BMP in mESCs. Stimulation of the ERK signaling pathway triggers the transition from pluripotency to lineage-commitment (Kunath et al., 2007); therefore, by blocking the ERK signaling pathway, lineage-commitment is inhibited and the ground state of ESC self-renewal is maintained. Suppression of ERK activation is achieved with the addition of PD0325901, and together with CHIR99021, have shown to be sufficient to sustain efficient ESC self-renewal (Ying et al., 2008).

We have previously shown that some sources of ASC, including BFF, express transcripts for the key pluripotency genes, Oct-4, Nanog, and Sox-2 (Coley, 2007). While these cell types' differentiating and self-renewing capabilities are far less robust than that of ESC, we reasoned that the same system involved in promoting the pluripotent state in mESC and hESC would similarly work to maintain, or possibly enhance, the less differentiated state characteristic of BFF cells. While there was no significant increase in Oct-4, Nanog, or Sox-2 relative gene expression detected between control and treatment samples at either day 7 or day 14, it is important to note that there was no significant decrease in the relative expression of these transcripts over time either. Typically, pluripotent gene expression in ASC decreases as passage number increases (Tsai and Hung, 2012), yet the extended culture period and consequential passaging required to maintain cells in culture for an extended period of time did not result in a significant decrease in Oct-4, Nanog, or Sox-2 expression.

This is an opportune time to point out that not all researchers agree that CHIR99021 and PD032591 facilitate somatic cell reprogramming. Several studies dissecting the mechanism of action of CHIR99021 and PD032591 in facilitating and enhancing nuclear reprogramming have been previously discussed in detail (Li et al.;
Buehr et al., 2008; Shi et al., 2008b; Ying et al., 2008; Li et al., 2009; Nichols and Smith, 2009; Hanna et al., 2010; Zhou et al., 2010; Tsutsui et al., 2011), and while there is no shortage of evidence supporting this, it is imperative to discuss those results conflicting with this notion. As previously discussed, the Wnt signaling pathway is postulated to play an important role in self-renewal in both human and mouse ESC. When the canonical Wnt signaling pathway is stimulated, β-catenin translocates to the nucleus where it interacts with Tcf/Lef proteins to activate target genes. With regards to nuclear reprogramming, the Wnt-β-catenin pathway is thought to help reestablish pluripotency specifically by alleviating the inhibitory effect of T-cell factor 3 (TCF3) on pluripotency (Grigoryan et al., 2008; Niwa, 2011). CHIR99021 has been shown to enable the production of iPSC from MEF cells through its' effect on Wnt signaling which leads to the overexpression of Oct-4 and Klf-4 (Li et al., 2009). The other school of thought is that the Wnt-β-catenin pathway actually induces cellular differentiation. A 2012 study by researchers at the Institute for Stem Cell and Regenerative Medicine and the Howard Hughes Medical Institute proposed a revised interpretation of the role of Wnt-β-catenin signaling in hESC, which supports a primary role for Wnt-β-catenin signaling in differentiation, rather than self-renewal of hESC in culture. Davidson and colleagues reported long-term expansion of hESC was possible with sustained inhibition of the Wnt-β-catenin signaling pathway, indicating that Wnt-β-catenin signaling is not required for undifferentiated hESC proliferation. Furthermore, they also found that Oct-4 functionally represses endogenous Wnt-β-catenin signaling in self-renewing hESC (Davidson et al., 2012). Lian and colleagues came to the same conclusion when they demonstrated that activation of the Wnt-β-catenin signaling pathway via CHIR990021
promoted the differentiation, not self-renewal, of hESC into cardiomyocytes (Lian et al., 2012).

More recently, the effects of PD0325091 and CHIR99021 on the induction of endogenous pluripotency genes in reprogrammed iPSC in porcine were investigated. Transfected porcine fetal fibroblast cells were treated with the two small molecule inhibitors (2i), and then examined for the activation of pluripotency markers in the fetal fibroblast cells during their conversion to iPSC by measuring alkaline-phosphatase (AP) expression. Researchers found that there were significantly fewer AP-positive colonies compared with cells cultured in medium supplemented only with LIF. Moreover, endogenous expression of pluripotency-related genes, including Oct-4, Nanog, and Sox-2, was significantly lower in 2i-treated cell cultures (Petkov et al., 2014). Other studies have similarly reported a decrease in the expression of key pluripotency genes, particularly Oct-4, in cells cultured with PD0325091 and CHIR99021 in porcine (Telugu et al., 2011; Gao et al., 2013). These findings cast doubt on the effectiveness of these small molecule inhibitors in the reprogramming of somatic cells to pluripotency in species other than the mouse and human, opening up the possibility that PD0325091 and CHIR99021 may be ineffective on bovine cells. While further research is necessary before any conclusions should be made, the results of these studies taken together with the findings of this study suggest that the cow may differ from the mouse and human in terms of pluripotent pathways.
CHAPTER IV
EPIGENETIC EFFECTS OF TREATMENT WITH SMALL MOLECULE INHIBITORS ON BOVINE FETAL FIBROBLAST CELLS

Introduction

The discovery of an increasing number of chemical compound that either induce or enhance nuclear reprogramming has revealed the basic mechanisms underlying the reprogramming process. Based on their mechanism of action, these chemical compounds fall broadly into several classes, including those that modulate (1) epigenetic protein activity, (2) signal transduction pathways, (3) transcription factor activity, and (4) cell metabolism (Li et al.). CHIR99021 is among those small molecules that modulate signal transduction pathways. CHIR99021 is a potent and selective inhibitor or GSK3, inhibiting both GSK3β and GSK3α. GSK3 is a serine/threonine kinase that is a key inhibitor of the Wnt signaling pathway. Because CHIR99021 inhibits GSK3, it functions as a Wnt activator (Bain et al., 2007). The Wnt signaling pathway plays an important role in self-renewal in both human and mouse ESC. When the canonical Wnt signaling pathway is stimulated, β-catenin translocates to the nucleus where it interacts with Tcf/Lef proteins to activate target genes. With regards to nuclear reprogramming, the Wnt-β-catenin pathway is thought to help reestablish pluripotency specifically by alleviating the inhibitory effect of T-cell factor 3 (TCF3) on pluripotency (Grigoryan et al., 2008; Niwa, 2011). CHIR99021 has been shown to enable the production of iPSC from MEF cells through its' effect on Wnt signaling which leads to the overexpression of Oct-4 and Klf-4 (Li et al., 2009). Likewise, CHIR99021 has been found capable of substituting for c-myc (Marson et al., 2008).
Successful nuclear reprogramming requires several highly-complex processes and mechanisms be carried out methodically. Gene expression profiling and RNA interference (RNAi) screening in MEF revealed three phases of nuclear reprogramming: initiation, maturation, and stabilization (Figure 4.1). Studies showed that the first phase, initiation, is characterized by a mesenchymal-to-epithelial transition (MET) that is driven by BMP signaling (Samavarchi-Tehrani et al., 2010), and that the repression of the pluripotency transgenes is required for the transition from the maturation phase to the stabilization phase (Li et al., 2010; Golipour et al., 2012). Genome-wide analysis of intermediate cells poised to become iPSC revealed that the reprogramming process encompasses two distinct waves of major gene activity. The first wave occurs between day 0 and day 3, and is characterized by the activation of genes responsible for proliferation, metabolism, cytoskeleton organization, and the downregulation of genes associated with development. The second wave of major gene activity occurs towards the end of the 12 day reprogramming process, after day 9, and is characterized by the expression of genes responsible for embryonic development and stem cell maintenance. It is at this stage that the core pluripotency-regulating network is activated and the pluripotent state is stabilized (Polo et al., 2012). Complimentary studies using single-cell techniques for quantifying gene expression in cells that undergo complete nuclear reprogramming towards pluripotency indicated that induction of reprogramming factors provokes stochastic gene expression changes in a subset of pluripotency genes early in the reprogramming process. These stochastic changes
Figure 4.1. Phases of the Reprogramming Process. In the model described by Buganim et al. (2013), the reprogramming process is divided into two phases: a long 'stochastic' phase of gene activation followed by a shorter hierarchical more 'deterministic' phase of gene activation that begins with the activation of the Sox2 locus. After induction, the four factor reprogramming cocktail, OSKM, initiates stochastic gene expression, and fibroblasts assume one of several possible fates (such as, apoptosis, senescence, transformation, transdifferentiation or reprogramming). In the early phase, reprogrammable cells will increase proliferation, undergo changes in histone modifications at somatic genes, initiate MET, and activate DNA repair and RNA processing (Samavarchi-Tehrani et al., 2010). The reprogrammable cells then enter an intermediate phase with an unknown rate-limiting step that delays the conversion to iPSCs and contributes to the long latency of the process. In this phase, cells undergo a stochastic activation of pluripotency markers, a transient activation of developmental regulators, and activation of glycolysis (Li et al., 2010; Golipour et al., 2012). In general the transcriptional changes in this phase are small. In some rare cases, the stochastic gene expression will lead to the activation of "predictive markers" such as Utf1, Esrrb, Dppa2, and Lin28, which ultimately activate Sox-2. Activation of Sox-2, either directly or indirectly by these markers, initializes the second phase of reprogramming by triggering a series of deterministic events that ultimately results in iPSC. In this late phase, the cells eventually stabilize into the pluripotent state as the transgenes are silenced, the cytoskeleton is remodeled to an ESC-like state, the epigenome is reset, and the core pluripotency circuitry is activated (Polo et al., 2012).
occur in tandem with MET and other events characteristic of the initiation phase, and are essential for cells to transition to the next phase (Li et al., 2010). Unlike the first phase, the second phase is believed to be a more deterministic phase of gene activation, in which the core pluripotency circuitry is activated and the pluripotent state is stabilized in cells. Very few cells make the transition to the second phase, however, as the majority become trapped in an intermediate phase between the first and second phases. The low efficiency of iPSC production is thought to be due to a rate-limiting stochastic event that is a precursor to the second phase of reprogramming (Buganim et al., 2012).

The epigenetic changes accompanying successful nuclear reprogramming include chromatin reorganization, DNA demethylation of promoter regions of key pluripotency-associated genes, reactivation of the somatically silenced X chromosome, and genome-wide resetting of histone posttranslational modifications (Takahashi et al., 2007; Maherali and Hochedlinger, 2009). While some of these processes, such as X chromosome reactivation, occur late in the reprogramming process, changes in histone modifications can be observed immediately after factor induction (Koche et al., 2011), suggesting that alterations in histone marks are an early event that is associated with initiation of the reprogramming process (Buganim et al., 2013). The roles of relevant histone marks during the reprogramming process are summarized in Table 4.1. The first of these changes to be observed is a peak of de novo deposition of H3K4me2 at the promoter and enhancer regions of many pluripotency-associated genes, including Sall4 and Fgf4, which are enriched for Oct-4 and Sox-2 binding sites and lack H3K4me1 or H3K4me3 marks. Concomitantly, the promoters of genes responsible for the somatic
### Table 4.1. Roles of Relevant Histone Marks during Nuclear Reprogramming (Buganim et al., 2013)

<table>
<thead>
<tr>
<th>Histone Mark</th>
<th>Function</th>
<th>Phase of Reprogramming in which Change Occurs</th>
<th>Example of Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3K4me2</td>
<td>Marks promoters and enhancers</td>
<td>Early phase</td>
<td>Decrease at MEF and EMT genes; increase at proliferation, metabolism, pluripotency, and MET genes</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>Marks active loci</td>
<td>Early phase</td>
<td>Increase at proliferation and metabolism genes</td>
</tr>
<tr>
<td>H3K27me3</td>
<td>Marks repressed loci</td>
<td>Early phase</td>
<td>Increase at MEF and EMT genes</td>
</tr>
<tr>
<td>H3K4me1</td>
<td>Marks enhancers</td>
<td>Early phase</td>
<td>Increase at proliferation and metabolism genes</td>
</tr>
<tr>
<td>H3K36me3</td>
<td>Marks transcriptionally active regions</td>
<td>Early to middle phase</td>
<td>Increase at early and late pluripotency genes</td>
</tr>
<tr>
<td>H3K9me3</td>
<td>Marks heterochromatin regions</td>
<td>Late phase</td>
<td>Decrease at late pluripotency genes</td>
</tr>
<tr>
<td>H3K36me2</td>
<td>Marks potential regulatory regions (such as newly transcribed genes)</td>
<td>Early phase</td>
<td>Increase at early pluripotency genes</td>
</tr>
<tr>
<td>H3K79me2</td>
<td>Marks transcriptionally active regions</td>
<td>Early to middle phase</td>
<td>Decrease at MEF and EMT genes</td>
</tr>
</tbody>
</table>
cell program being to lose H3K4me2 (Koche et al., 2011). Chromatin reorganization from the somatic state to an ESC-like one is required for the activation of the pluripotency circuitry system. It appears that the chromatin reorganization events take place in a coordinated and sequential manner. Rearrangement of the heterochromatin, characterized by the presence of H3K9me3 and heterochromatin protein 1, precedes the activation of Nanog, while enrichment of euchromatin marks occurs concurrently with Nanog activation (Mattout et al., 2011). Taking into consideration the epigenetic changes in histones in iPSC, H3 is the histone researched the most, as it is directly related to genes expressed during embryonic development, such as Oct-4, Nanog, and Sox-2. Methylation of H3K27 is associated with the suppression of various genes, and persistent H3K27me3 blocks reprogramming. However, methylation of H3K4 is associated with the activation of different embryonic genes. Termed "bivalent domains", these are regions enriched for repressive H3K27me3 and simultaneously for activating H3K4me3 (Mikkelsen et al., 2007). Bivalent domains were initially thought to be ESC-specific, however, Mikkelsen and colleagues (2007) showed that bivalent domains are more indicative of genes that exist in a poised state. Genes that harbor the bivalent domain are transcriptionally silenced in ESCs, suggesting a potentially dominant role of H3K27me3 (Maherali and Hochedlinger, 2009). In ESCs genes with bivalent domain include a substantial number of differentiation-related genes targeted by the core pluripotency factors. The bivalent domain on the promoters of these genes allows for a quick response to differentiation cues. As ESC begin to differentiate, most bivalent genes lose one of the marks, leaving either the repressive mark H3K27me3 or the activating mark H3K4me3 (Mikkelsen et al., 2007; Pan et al., 2007; Zhao et al., 2007). It
should be noted that the significance of bivalent domains in key developmental genes has recently been brought into question. Bivalent domains are not a universal feature of pluripotent cells, nor are they unique to pluripotent cells (Mikkelsen et al., 2007). Mouse ES cells can be maintained in a naïve state in the absence of serum by MEK and GSK3 inhibitors (2i). A recent study by Marks and colleagues showed that mouse ESC grown in 2i medium, compared to serum-cultured mouse ESC, exhibit highly similar H3K4me3 profiles, but substantially reduced prevalence of H3K27me3, resulting in significantly fewer bivalent domains. In spite of this, these genes are still effectively silenced (Marks et al., 2012). Therefore, another mechanism must contribute to the repression of these loci, and a large proportion of bivalent domains in ES cells cultured in serum are due to acquisition of H3K27me3 at promoters (Marks et al., 2012; Liang and Zhang, 2013; Chen and Dent, 2014).

H3K9 methylation is associated with transcription silencing and heterochromatin formation. Genome-wide localization studies have shown that the genomic domains marked with H3K9me3 are substantially expanded in differentiated cells compared with ESC (Hawkins et al., 2010). H3K9 methylation has been identified as the key barrier to the acquisition of pluripotency following nuclear reprogramming, as it has been shown to determine cell fate between pre-iPSC and iPSC. It is thought that the switch between euchromatin and heterochromatin is dependent upon the balance between H3K9 methylation and demethylation (Chen et al., 2013). As ESC differentiate, G9a, a H3K9-specific HMT, contributes to silencing the Oct-4 locus by establishing a heterochromatin state. Conversely, H3K9 histone demethylases (HDM) are essential to maintaining ESC identity (Loh et al., 2007). Although H3K9 methylation has been dubbed the
primary hindrance to fully reprogrammed iPSC, mechanistic insights into the role H3K9 methylation plays in the epigenetic reprogramming of cell fate lends H3K9 methylation to be a tangible barrier to reprogramming, capable of being overcome. This decreasing the levels of H3K9 methyltransferases or overexpressing H3K9 demethylases enhances the efficiency of iPSC production (Kubicek et al., 2007; Loh et al., 2007; Shi et al., 2008a; Shi et al., 2008b; Huang et al., 2010).

Unlike ESC which are detected only in the ICM of a blastocyst, adult stem cells (ASC) are found in a variety of adult mammalian tissues and organs. ASC primarily function to replenish damaged cells within these tissues and organs as a result of normal cellular senescence or injury (Odorico et al., 2001). The differentiation potential ASC has long been thought to be limited to cell lineages present in the organ from which they are derived; however, several studies have challenged this notion by demonstrating that some ASC exhibit a particularly high degree of plasticity. Unlike terminally differentiated somatic cells, the less differentiated state of ASC can assume the functional phenotypes and expression profiles of cells unique to other tissues (Herzog et al., 2003). Furthermore, we and other groups have reported the presence of Oct-4, Nanog, and Sox-2 expression in some sources of ASC in the cow, mouse, and pig (Kues et al., 2005; Carlin et al., 2006; Coley, 2007). These findings support the notion that ASC less differentiated than other somatic cell types, and share some characteristics with ESC, including epigenomic regulatory program. While the chromatin of ASC is globally less in an open configuration than that of ESC, a common set of “stemness genes”, including regulators of chromatin, transcription, cell cycle and survival is marked by H3K4me3, and is active in both mouse hair follicle stem cells (HF-
SC) and ESC (Lien et al., 2011). In hematopoietic stem cells (HSC), H3K4me3 is more prevalent compared to differentiated cells types, and enhancers of differentiation genes are marked by monomethylation of H3K4, H3K9, and H3k27, which is likely involved in maintaining activation potential required for differentiation (Cui et al., 2009).

Once established, cellular identity is remarkably stable. Chromatin modifications are faithfully inherited from cell division to cell division, highlighting the major hurdles iPSC must overcome in order to be fully reprogrammed to pluripotency. The use of small molecule compounds that target chromatin modifications and/or specific signaling pathways have proven to be effective at overcoming these reprogramming hurdles. Considering the significance of H3K4, H3K9, and H3K27 methylation in the determination of cell fate, we sought to assess the effects of pre-treatment with small molecules on these histone modifications at the promoter regions of Oct-4, Nanog, and Sox-2.

**Materials and Methods**

Experimental Design

BFF cells were cultured in the presence of three small molecule inhibitors (3i): PD0325901, a mitogen activated protein kinase/ERK kinase (MEK) inhibitor; CHIR99021, a glycogen synthase kinase-3 (GSK3) inhibitor; and NuP0178 (NuPotential, Baton Rouge, LA), a G9a histone methyltransferase inhibitor, for a total of 14 days. On day 7 and day 14, cells were fixed using 37% formaldehyde solution, and harvested for the preparation of chromatin using sonication shearing. The resulting chromatin was then immunoprecipitated with antibodies directed against H3K4me3,
H3K9me3, and H3K27me. Following immunoprecipitation, DNA was recovered and analyzed by qRT-PCR to identify DNA loci associated with H3K4me3, H3K9me3, and H3K27me3 on Oct-4, Nanog, and Sox-2.

Establishment of Cell Lines and Maintenance

Target cell lines were obtained from previously isolated primary cultures. Primary cultures of fibroblast cells were established from bovine fetuses approximately 50 days old according to protocol (Giraldo et al., 2009). These Bovine Fetal Fibroblast (BFF) cells were cultured in complete culture medium composed of Dulbecco’s Modified Eagle Medium (DMEM) with high glucose, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (P/S). Once cells reached 80% confluency, cultures were passaged by releasing cells with trypsin (0.25%). BFF cells were then resuspended in DMEM supplemented with 10% bovine calf serum (CS) and 10% dimethyl sulfoxide (DMSO) for cryopreservation. Cryovials containing approximately 1,000,000 BFF cells suspended in solution were cooled at 1°C/min until reaching -80°C before storage in liquid nitrogen.

BFF cells were thawed as needed at room temperature for 30 sec, followed by submersion in a 38°C water bath. Cells were then washed in complete culture medium and seeded at a density of 0.7 x 10^6 into 25-cm² flasks and cultured under 5% CO₂ and 90% humidity at 37°C. Because the experimental procedure required a high volume of cells, BFF cells were expanded through repeated passaging until a sufficient number of cells were obtained prior to each experiment.
Treatment with Small Molecules

Chemical treatment consisted of 3 small molecule inhibitors (3i): PD0325901, a mitogen activated protein kinase/ERK kinase (MEK) inhibitor; CHIR99021, a glycogen synthase kinase-3 (GSK3) inhibitor; and NuP0178 (NuPotential, Baton Rouge, LA), a G9a histone methyltransferase inhibitor. A 10mM stock solution of each inhibitor was prepared by dissolving the crystalline solid in DMSO. 3i+ media was prepared by diluting each stock into complete culture medium (DMEM, 10% FBS, 1% P/S) to a concentration of 0.5µM PD0325901; 3µM CHIR99021; 1.8µM NuP0178. The concentrations of CHIR99021 and PD0325901 utilized were consistent with previous studies (Yu et al., 2011) and NuP0148 concentration was estimated due to its recent development and concentration evaluation experiments carried out by NuPotential, LLC.

Once BFF cells were expanded to an adequate quantity, trypsin (0.25% EDTA) was added to confluent cultures of BFF cells to disaggregate cells adherent to the flask, which were then counted using a hemocytometer. Cells were then divided evenly into treatment and control groups, approximately 2.5 x 10^6 cells per group. Control cells were resuspended in complete culture medium and split evenly between three 25-cm² flasks (approximately 0.7 x 10^6 per flask). Similarly, treatment cells were resuspended in 3i+ media and split evenly between three 25-cm² flasks (approximately 0.7 x 10^6 per flask). All flasks were then placed in 37°C incubator with 5% CO₂ and 90% humidity. Media was changed every 1-2 days, and cells were passaged as needed.
Cell Fixation and Shearing

On days 7 and 14, cells grown in a T-75 culture flask were harvested for chromatin immunoprecipitation (ChIP) procedures using ChIP-IT® Express Magnetic Chromatin Immunoprecipitation Kit & Sonication Shearing Kit (Active Motif, 53008). The existing culture medium was removed from flasks, and 20 ml of freshly-prepared Fixation Solution, composed of 37% formaldehyde and complete culture medium (540 µl 37% formaldehyde + 20 ml complete culture medium), was added to fix protein/DNA interactions. Cells were incubated on a shaking platform for 10 min at RT, and then washed in ice-cold 1X PBS solution. To stop the fixation reaction, 10 ml Glycine Stop Fix Solution (1 ml 10X Glycine Buffer + 1 ml 10X PBS + 8 ml dH2O) was added to each flask and incubated on a shaking platform for 5 min at RT. Cells were washed in ice-cold 1X PBS solution, and 5 ml of ice-cold Cell Scraping Solution (dH2O with 10% 10X PBS) with 30 µl 100 mM PMSF was added to each flask. Adherent cells were dislodged by thoroughly scraping the bottom of the culture flask with a rubber policeman. Cells were collected in a 15 ml conical tube held on ice, and then pelleted by centrifugation at 720 x g for 10 min at 4°C. After discarding the supernatant, 1 µl 100 mM PMSF and 1 µl PIC were added to the pellet and placed in -80°C freezer until use in sonication step.

Due to the nature of the experimental condition, growth and survival of cell cultures for an extended period of time proved to be quite difficult. We found that cells did best when they were maintained in smaller culture vessels (T-25) throughout the experiment. In this case, cells were harvested on day 7 and 14 by trypsinization, and washed in 1 ml of Dulbecco’s Phosphate Buffered Solution (DPBS) with Ca²⁺ and Mg²⁺. Cells were then resuspended in Fixation Solution (1 ml per 1-2 x 10⁶ cells) and allowed
to incubate for 10 min. In-suspension fixation was stopped by the addition of 10% 10X Glycine. Cells were pelleted by centrifugation at 720 x g for 5 min at 4°C, and immediately washed in ice-cold 1X PBS solution. After discarding the supernatant, 1 µl 100 mM PMSF and 1 µl PIC were added to the pellet and placed in -80°C freezer until use in sonication step.

Optimization of Chromatin Shearing by Sonication

Chromatin shearing conditions can vary greatly depending on the cell type and the treatment in the experiment; therefore, shearing conditions must be optimized for these factors specific to this experiment. To do this, BFF cells were grown to 70-80% confluency in two 15 cm plates under standard cell culture conditions (DMEM, 10% FBS, 1% P/S; 37°C, 5% CO$_2$). When the cells were ready to be harvested, the existing culture medium was aspirated from the plates, and 20 ml of freshly-prepared Fixation Solution, composed of 37% formaldehyde and complete culture medium (540 µl 37% formaldehyde + 20 ml complete culture medium), was added to each plate to fix protein/DNA interactions. The plates were placed on a shaking platform for 10 min at RT, and then washed in 10 ml ice-cold 1X PBS solution. The fixation reaction was stopped by adding 10 ml Glycine Stop-Fix Solution (1 ml 10X Glycine Buffer+ 1 ml 10X PBS + 8 ml dH$_2$O) to each plate. The plates were incubated on a shaking platform for 5 min at RT, and then washed in 10 ml ice-cold 1X PBS solution. Immediately before use, 60 µl 100 mM PMSF was added to 12 ml of ice-cold Cell Scraping Solution (5.4 ml dH$_2$O + 600 µl 10X PBS). 5 ml of Cell Scraping Solution was added to each plate, and cells were scraped down the plate using a rubber policeman. Cells from both plates were collected and pooled together in a 15 ml conical tube and pelleted by
After aspirating the supernatant, 1 µl 100 mM PMSF and 1 µl PIC was added to the cell pellet and placed in a -80°C freezer overnight in order to best replicate sample conditions. The next day, the cell pellet was thawed on ice and resuspended in 3 ml ice-cold Lysis Buffer (ChIP-IT Express Kit, Active Motif 53008) supplemented with 15 µl PMSF and 15 µl PIC. Following a 30 min incubation on ice, cells were transferred (1 ml at a time) to a dounce homogenizer and lysed on ice with 30 strokes. The cell lysate was collected in a 15 ml conical tube and pelleted by centrifugation. The supernatant was discarded, and the cell pellet was resuspended in 2 ml Shearing Buffer (ChIP-IT Express Kit, Active Motif 53008) supplemented with 10 µl PMSF and 10 µl PIC. The cell suspension was aliquoted into equal volumes in six 1.7 ml microcentrifuge tubes, and the tubes were placed on ice. The six aliquots of fixed chromatin were sheared using Cole Parmer Ultrasonic Processor (Cole-Parmer, Vernon Hills, IL, USA) (500 watts, 120 volts, 3 mm probe) at 30% amplitude using six different conditions: (1): 20 pulses of 20 sec, 30 sec rest; (2): 30 pulses of 20 sec, 30 sec rest; (3): 40 pulses of 20 sec, 30 sec rest; (4): 20 pulses of 30 sec, 30 sec rest; (5): 30 pulses of 30 sec, 30 sec rest; (6): 40 pulses of 30 sec, 30 sec rest. After sonication, the tubes were centrifuged for 10 min at 14000 x g at 4°C, and the supernatant, containing the sheared chromatin, was collected and prepared into 50 µl aliquots. One 50 µl aliquot of sheared chromatin from each of the six shearing conditions was subjected to cross-link reversal and purified according to protocol described below. Samples were separated by electrophoresis through a 1% agarose gel to determine the optimal shearing conditions. Optimally sheared chromatin will yield a smear between 200-1500 bp.
Based on this assay, we concluded that shearing using 25 pulses of 20 sec, 30 sec rest yielded chromatin suitable for our experimentations.

DNA Clean Up to Assess Shearing Efficiency and DNA Concentration

To assess shearing efficiency as well as determine the DNA concentration, 150 µl of dH₂O was added to 50 µl of each sheared chromatin sample, followed by 10 µl 5 M NaCl. The tubes were then placed in a thermocycler at 65°C overnight to reverse the cross-links. The following day, the samples were removed from the thermocycler and 1 µl RNase A (10 µg/µl) was added to each tube. The samples were then incubated for 15 min at 37°C. Next, 10 µl Proteinase K (0.5 µg/µl) was added to each sample, and the tubes were placed in a thermocycler at 42°C to incubate for 1.5 h. After incubation, DNA was cleaned up by adding 200 µl 1:1 phenol/chloroform TE saturated pH 8 (Sigma Aldrich, P3803) to each sample, vortexed, and centrifuged for 5 min at 14000 x g. The aqueous phase was then transferred to a new microcentrifuge tube, and 20 µl 3 M Sodium Acetate pH 5.2 (Sigma-Aldrich, S2889) followed by 500 µl 100% ethanol was added to each sample. 1 µl glycogen (20 µg/µl) (ThermoFisher Scientific, 10814010) was also added to each sample to act as carrier for DNA in order to improve recovery. The tubes were vortexed and placed in -20°C freezer overnight to permit DNA precipitation. The next day, the tubes were centrifuged for 10 min at 14000 x g at 4°C to separate the precipitate from the supernatant. After removing the supernatant, the pellet was washed in 500 µl 70% ice cold ethanol and centrifuged again at 14000 x g for 5 min in a 4°C microcentrifuge. The supernatant was carefully removed, and the pellet was allowed to air-dry before resuspension in 30 µl dH₂O. DNA concentration was
measured using a Thermo Scientific™ NanoDrop 2000 Spectrophotometer (Wilmington, DE, USA).

Shearing by Sonication

Previously frozen pellets of cross-linked cells were thawed on ice, resuspended in 1 ml of ice-cold Lysis Buffer supplemented with 5 µl PIC + 5 µl PMSF, and held on ice for 30 min to initiate cell lysis. The cell suspension was then transferred to a glass pestle on ice for Dounce Homogenization. Cell lysis was monitored under phase contrast microscopy after every 10 strokes until the nuclei were sufficiently released (between 20 and 40 strokes). The cells were then transferred to a 1.7 microcentrifuge tubes and centrifuged at 720 x g for 10 min at 4°C. After discarding the supernatant, the nuclei pellet was resuspended in 300 µl Shearing Buffer (ChIP-IT Express Kit, Active Motif 53008) supplemented with 1.5 µl PIC and 1.5 µl PMSF. DNA was sheared using Cole Parmer Ultrasonic Processor (Cole-Parmer, Vernon Hills, IL, USA) (500 watts, 120 volts, 3 mm probe) under the optimal conditions determined for the BFF cells used in this experiment (25 pulses of 20 sec, 30 sec rest). After sonication, the sheared chromatin was separated from the cellular debris by centrifugation at 14000 x g for 10 min at 4°C and prepared into 50 µl aliquots. One aliquot was immediately used for assessing DNA shearing efficiency and determining the DNA concentration. The remaining aliquots of sheared chromatin were stored at -80°C for later use.

Chromatin Immunoprecipitation

Previously frozen aliquots of sheared chromatin were thawed on ice. Once thawed, 10 µl was removed and processed as “Input DNA”. Chromatin
Immunoprecipitation (ChIP) reactions were set up in siliconized 1.7 ml microcentrifuge tubes using reagents provided in ChIP-IT® Express Kit (Active Motif, 53052), with the exception of the antibodies. Antibodies for H3K4me3 (Active Motif, 39915), H3K9me3 (Active Motif, 39765), H3K27me3 (Active Motif, 39155) were reconstituted to a concentration of 1 µg/µl and added as the final component of the ChIP reaction. First, the volume of sheared chromatin needed for each reaction was calculated based on the DNA concentration that was previously determined. For reactions using less than 60 µl of chromatin, 25 µl Protein G Magnetic Beads, 10 µl ChIP Buffer 1, 1 µl PIC, and 3 µg of antibody were added to the appropriate volume of sheared chromatin and brought up to a final volume of 100 µl with dH2O. For reactions requiring more than 60 µl of chromatin, 25 µl Protein G Magnetic Beads, 20 µl ChIP Buffer 2, 2 µl PIC, and 3 µg of antibody were added to the appropriate volume of sheared chromatin and brought up to a final volume of 200 µl with dH2O. Chromatin was immunoprecipitated by incubating the reactions on an end-to-end rotator overnight at 4°C. The beads were separated from the supernatant using a magnetic stand, and quickly washed once in 800 µl ChIP Buffer 1, followed by two washes in 800 µl ChIP Buffer 2. Chromatin was eluted by incubating the beads in 50 µl Elution Buffer AM2 for 15 min at RT on an end-to-end rotator. 50 µl Reverse Cross-linking Buffer was mixed with the eluted chromatin. After the beads were separated, the supernatant was transferred to a fresh PCR tube, and both the ChIP and Input DNA samples were incubated at 95°C for 15 min. 2 µl Proteinase K (0.5 µg/µl) was added to each tube and successively incubated at 37°C for 1 h. Lastly, 2 µl Proteinase K Stop Solution was added to each sample.
Chromatin Immunoprecipitation DNA Purification

Before ChIP samples can be used in PCR, the DNA must be purified away from the components and contaminants present in an eluted ChIP sample. Chromatin IP DNA Purification Kit (Active Motif, 58002) was used to purify DNA in all ChIP samples. To each sample, 5 volumes DNA Purification Binding Buffer was added for every one volume of sample. Next, 5 µl 3 M Sodium Acetate (pH 5.2) was added to determine the solution had a pH less than 7.5. Samples were then briefly centrifuged, and the DNA was captured by a DNA Purification Column. Column-bound DNA was washed with 750 µl Wash Buffer, and the flow through was discarded. Following another brief spin in order to remove any residual Wash Buffer from the column, the column was transferred to a new 1.7 microcentrifuge tube, and the DNA was eluted using 50 µl DNA Purification Elution Buffer.

Quantitative Real-Time PCR

The methylation status of the promoter regions of Oct-4, Nanog, and Sox-2 in both untreated and 3i+ BFF cells at day 7 and 14 was analyzed using Bio-Rad CFX.

**Table 4.2.** Primer sets for qRT-PCR for Quantification of Target Enrichment

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct-4</td>
<td>Forward TGGGTCGAGGGTTAGAGT&lt;br&gt;Reverse CAACAACTCAGTCTCCTC</td>
</tr>
<tr>
<td>Nanog</td>
<td>Forward AGGGATTGAAGTTTAAAAAAA&lt;br&gt;Reverse TATCCAAACATCCAAAAATAAAA</td>
</tr>
<tr>
<td>Sox-2</td>
<td>Forward GCGTTTTTTTTTTTTTTTTTTTAGTAGT&lt;br&gt;Reverse ACTTTCCCCCTTTTACAAACA</td>
</tr>
</tbody>
</table>
Connect™ Real-Time PCR Detection System. The PCR primers designed to amplify the promoter regions of these genes were previously validated by (Huang et al. (2014)). We confirmed the specificity of these primer sequences (Table 4.2) through RT-PCR followed by gel electrophoresis using calibrator cDNA produced from a pool of bovine genomic DNA. Reactions were carried out in a total volume of 20 µl, which consisted of 10 µl SsoFast™ EvaGreen® Supermix (Bio Rad, 1725201), 1 µl forward and reverse primer pairs (10 mM), 6 µl DEPC H₂O, and 2 µl sample DNA. Reaction mixes were concocted for each ChIP DNA sample, Input DNA, No Ab control sample, and a no template negative control for all three genes. The PCR program used for the amplification of all genes consisted of a denaturing cycle of 2 min at 94°C; 40 cycles of PCR (94°C for 30 sec, 52°C for 40 sec, 72°C for 30 sec, 72°C for 7 min).

ChIP-qPCR data must be normalized for sources of variability, including amount of chromatin, efficiency of immunoprecipitation, and DNA recovery. Data in this study was normalized using the Percent of Input Method. The Percent of Input Method assumes that the ChIP qPCR signals are directly related to the amount of input chromatin. Therefore, these values must be adjusted for using the following equation:
\[
\Delta C_t \text{ [normalized ChIP]} = (C_t \text{ [ChIP]} - (C_t \text{ [Input]} - \log_2 \text{(Input Dilution Factor)}),
\]
where Input Dilution Factor = (fraction of the input chromatin saved)^{-1}. Once the adjusted input value was calculated for each gene in each sample, the normalized ΔCt for each ChIP sample was calculated. Finally, the percent of input (Input %) for each sample is calculated as follows: Input % = 100/2^{\Delta C_t \text{ [normalized ChIP]}}. Data in this study is presented as a percentage of the input, representing the enrichment of precipitated DNA associated with either H3K4me3, H3K9me3, or H3K27me3 relative to the input sample.
Statistical Analysis

In this experiment, a completely random design with repeated measures was employed to assess any effect treatment with 3i+ media had on the methylation status of H3K4, H3K9, and H3K27 at Oct-4, Nanog, and Sox-2 promoter regions over time. The PROC MIXED with repeated measures analysis with autoregression of order one covariance was used in SAS statistical software to determine any statistical differences H3K4, H3K9, and H3K27 enrichment at Oct-4, Nanog, and Sox-2 promoters relative to the input between treatment and control groups over time.

Results

The PROC MIXED with repeated measures analysis with autoregression of order one covariance was used to determine the effect of treatment, time, and the treatment*time interaction has on trimethylation of H3K4, H3K9, and H3K27 on the promoters of Oct-4, Nanog, and Sox-2 in untreated BFF cells and 3i+ treated BFF cells over time (Tables 4.2. – 4.7). For each effect, p-value ≤ 0.05 indicates a significant difference exists. The treatment* time interaction was found to have a significant effect on the methylation status of H3K4 in Nanog (p = 0.02), as well as H3K9 in Oct-4 (p = 0.05). Additionally, time was determined to have a significant effect on H3K27 methylation in Sox-2 (p = 0.04) (Figures 4.2 – 4.4). No significant difference in enrichment was detected in Oct-4 H3K4, Oct-4 H3K27, Nanog H3K9, Nanog H3K27, Sox-2 H3K4, Sox-2 H3K9 as an effect of treatment, time, or treatment*time interaction.
Table 4.3. H3K4me3, H3K9me3, H3K27me3 Enrichment at Oct-4 promoter in Control and Treatment BFF cells on Day 7

<table>
<thead>
<tr>
<th>Sample</th>
<th>Oct-4 H3K4me3</th>
<th>Oct-4 H3K9me3</th>
<th>Oct-4 H3K27me3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEZ 3 P4 CON (A)</td>
<td>0.0059%</td>
<td>0.0296%</td>
<td>0.0449%</td>
</tr>
<tr>
<td>BEX 1 P3 CON</td>
<td>0.0631%</td>
<td>0.7239%</td>
<td>0.4944%</td>
</tr>
<tr>
<td>BEZ 3 P4 CON (B)</td>
<td>0.0110%</td>
<td>0.0089%</td>
<td>0.0126%</td>
</tr>
<tr>
<td>BEX 2 P5 CON</td>
<td>0.7867%</td>
<td>2.80%</td>
<td>1.30%</td>
</tr>
<tr>
<td>BEX 3 P6 CON</td>
<td>1.09%</td>
<td>0.9618%</td>
<td>0.0977%</td>
</tr>
<tr>
<td>BEZ 1 P2 CON</td>
<td>9.21%</td>
<td>11.19%</td>
<td>5.87%</td>
</tr>
<tr>
<td>BEZ 2 P4 TRMT</td>
<td>1.05%</td>
<td>1.73%</td>
<td>1.07%</td>
</tr>
<tr>
<td>BEX 4 P3 TRMT</td>
<td>0.5373%</td>
<td>0.6399%</td>
<td>0.2307%</td>
</tr>
<tr>
<td>BEZ 1 P2 TRMT (A)</td>
<td>3.40%</td>
<td>2.19%</td>
<td>3.82%</td>
</tr>
<tr>
<td>BEZ 1 P2 TRMT (B)</td>
<td>0.0546%</td>
<td>0.1797%</td>
<td>0.0761%</td>
</tr>
</tbody>
</table>
### Table 4.4. H3K4me3, H3K9me3, H3K27me3 Enrichment at Nanog promoter in Control and Treatment BFF cells on Day 7

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nanog H3K4me3</th>
<th>Nanog H3K9me3</th>
<th>Nanog H3K27me3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEZ 3 P4 CON (A)</td>
<td>8.96%</td>
<td>0.2860%</td>
<td>2.24%</td>
</tr>
<tr>
<td>BEX 1 P3 CON</td>
<td>0.2291%</td>
<td>27.55%</td>
<td>13.77%</td>
</tr>
<tr>
<td>BEZ 3 P4 CON (B)</td>
<td>177.77%</td>
<td>1290.63%</td>
<td>1041.07%</td>
</tr>
<tr>
<td>BEX 2 P5 CON</td>
<td>5.91%</td>
<td>2.13%</td>
<td>19.50%</td>
</tr>
<tr>
<td>BEX 3 P6 CON</td>
<td>1.34%</td>
<td>0.7494%</td>
<td>0.4016%</td>
</tr>
<tr>
<td>BEZ 1 P2 CON</td>
<td>2.92%</td>
<td>1.75%</td>
<td>5.29%</td>
</tr>
<tr>
<td>BEZ 2 P4 TRMT</td>
<td>8.13%</td>
<td>1.54%</td>
<td>3.15%</td>
</tr>
<tr>
<td>BEX 4 P3 TRMT</td>
<td>3.49%</td>
<td>1.00%</td>
<td>1.46%</td>
</tr>
<tr>
<td>BEZ 1 P2 TRMT (A)</td>
<td>52.12%</td>
<td>59.05%</td>
<td>34.63%</td>
</tr>
<tr>
<td>BEZ 1 P2 TRMT (B)</td>
<td>0.8373%</td>
<td>3.30%</td>
<td>8.19%</td>
</tr>
</tbody>
</table>
Table 4.5. H3K4me3, H3K9me3, H3K27me3 Enrichment at Sox-2 promoter in Control and Treatment BFF cells on Day 7

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sox-2 H3K4me3</th>
<th>Sox-2 H3K9me3</th>
<th>Sox-2 H3K27me3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEZ 3 P4 CON (A)</td>
<td>331.73%</td>
<td>42.93%</td>
<td>2.56%</td>
</tr>
<tr>
<td>BEX 1 P3 CON</td>
<td>437.72%</td>
<td>1.19%</td>
<td>0.4216%</td>
</tr>
<tr>
<td>BEZ 3 P4 CON (B)</td>
<td>4.10%</td>
<td>35.60%</td>
<td>0.4809%</td>
</tr>
<tr>
<td>BEX 2 P5 CON</td>
<td>0.2650%</td>
<td>0.6480%</td>
<td>0.2405%</td>
</tr>
<tr>
<td>BEX 3 P6 CON</td>
<td>0.2438%</td>
<td>0.2022%</td>
<td>0.05601%</td>
</tr>
<tr>
<td>BEZ 1 P2 CON</td>
<td>0.0772%</td>
<td>0.2920%</td>
<td>1.02%</td>
</tr>
<tr>
<td>BEZ 2 P4 TRMT (A)</td>
<td>1.71%</td>
<td>0.1712%</td>
<td>19.34%</td>
</tr>
<tr>
<td>BEX 4 P3 TRMT</td>
<td>0.3826%</td>
<td>0.8373%</td>
<td>0.0880%</td>
</tr>
<tr>
<td>BEZ 1 P2 TRMT (A)</td>
<td>0.8201%</td>
<td>4.51%</td>
<td>0.7922%</td>
</tr>
<tr>
<td>BEZ 1 P2 TRMT (B)</td>
<td>0.1084%</td>
<td>1.18%</td>
<td>0.2438%</td>
</tr>
</tbody>
</table>
Table 4.6. H3K4me3, H3K9me3, H3K27me3 Enrichment at Oct-4 promoter in Control and Treatment BFF cells on Day 14

<table>
<thead>
<tr>
<th>Sample</th>
<th>Oct-4 H3K4me3</th>
<th>Oct-4 H3K9me3</th>
<th>Oct-4 H3K27me3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEZ 2 P5 CON</td>
<td>0.1011%</td>
<td>0.2022%</td>
<td>3.04%</td>
</tr>
<tr>
<td>BEZ 3 P5 CON (A)</td>
<td>1.18%</td>
<td>0.6992%</td>
<td>0.0022%</td>
</tr>
<tr>
<td>BEZ 1 P4 CON</td>
<td>0.0622%</td>
<td>0.4245%</td>
<td>0.1712%</td>
</tr>
<tr>
<td>BEZ 2 P5 TRMT</td>
<td>3.35%</td>
<td>8.30%</td>
<td>3.37%</td>
</tr>
<tr>
<td>BEZ 3 P5 TRMT</td>
<td>6.12%</td>
<td>22.38%</td>
<td>34.15%</td>
</tr>
<tr>
<td>BEX 4 P6 TRMT</td>
<td>5.18%</td>
<td>4.77%</td>
<td>4.77%</td>
</tr>
</tbody>
</table>

Table 4.7. H3K4me3, H3K9me3, H3K27me3 Enrichment at Nanog promoter in Control and Treatment BFF cells on Day 14

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nanog H3K4me3</th>
<th>Nanog H3K9me3</th>
<th>Nanog H3K27me3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEZ 2 P5 CON</td>
<td>175.32%</td>
<td>118.92%</td>
<td>3.35%</td>
</tr>
<tr>
<td>BEZ 3 P5 CON (A)</td>
<td>16.27%</td>
<td>1.36%</td>
<td>7.80%</td>
</tr>
<tr>
<td>BEZ 1 P4 CON</td>
<td>7.38%</td>
<td>2.32%</td>
<td>11.91%</td>
</tr>
<tr>
<td>BEZ 2 P5 TRMT</td>
<td>3.42%</td>
<td>2.68%</td>
<td>1.80%</td>
</tr>
<tr>
<td>BEZ 3 P5 TRMT</td>
<td>723110.30%</td>
<td>184576.10%</td>
<td>616549.07%</td>
</tr>
<tr>
<td>BEX 4 P6 TRMT</td>
<td>1.96%</td>
<td>65.98%</td>
<td>93.30%</td>
</tr>
<tr>
<td>Sample</td>
<td>Sox-2 H3K4me3</td>
<td>Sox-2 H3K9me3</td>
<td>Sox-2 H3K27me3</td>
</tr>
<tr>
<td>-------------------</td>
<td>---------------</td>
<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
<td>BEZ 2 P5 CON</td>
<td>5.83%</td>
<td>3.24%</td>
<td>9.54%</td>
</tr>
<tr>
<td>BEZ 3 P5 CON (A)</td>
<td>6.60%</td>
<td>3.82%</td>
<td>4.09%</td>
</tr>
<tr>
<td>BEZ 1 P4 CON</td>
<td>0.8032%</td>
<td>0.2860%</td>
<td>0.0930%</td>
</tr>
<tr>
<td>BEZ 2 P5 TRMT</td>
<td>1.16%</td>
<td>16.04%</td>
<td>69.74%</td>
</tr>
<tr>
<td>BEZ 3 P5 TRMT</td>
<td>1.26%</td>
<td>7.13%</td>
<td>0.3002%</td>
</tr>
<tr>
<td>BEX 4 P6 TRMT</td>
<td>3.37%</td>
<td>100.69%</td>
<td>27.74%</td>
</tr>
</tbody>
</table>
**Figure 4.2.** ChIP followed by qRT-PCR was used to examine H3K4me3, H3K9me3, and H3K27me3 enrichment at the promoter region of Oct-4 in BFF cells treated with and without PD0325901, CHIR99021, and NuP0178 (3i) at day 7 and day 14. Enrichment is expressed as a ratio of immunoprecipitated DNA over input DNA. Percent of Input was calculated using the following equation: Input % = 100/2^{ΔCt [normalized ChIP]}, where ΔCt [normalized ChIP] = (Ct [ChIP] - (Ct [Input] - Log2 (Input Dilution Factor). This data is presented in Table 4.2. and Table 4.5. The interaction between time and treatment was observed to have a significant effect on H3K9me3 enrichment at the Oct-4 promoter (p = 0.05), and is illustrated above.
Figure 4.3. ChIP followed by qRT-PCR was used to examine H3K4me3, H3K9me3, and H3K27me3 enrichment at the promoter region of Nanog in BFF cells treated with and without PD0325901, CHIR99021, and NuP0178 (3i) at day 7 and day 14. Enrichment is expressed as a ratio of immunoprecipitated DNA over input DNA. Percent of Input was calculated using the following equation: \( \text{Input} \% = \frac{100}{2^{\Delta \text{Ct} \ [\text{normalized ChIP}]}} \), where \( \Delta \text{Ct} \ [\text{normalized ChIP}] = (\text{Ct [ChIP]} - (\text{Ct [Input]} - \log_2 \text{Input Dilution Factor}) \). This data is presented in Table 4.2. and Table 4.5. The interaction between time and treatment was observed to have a significant effect on H3K4me3 enrichment at the Nanog promoter (\( p = 0.02 \)), and is illustrated above.
Figure 4.4. ChIP followed by qRT-PCR was used to examine H3K4me3, H3K9me3, and H3K27me3 enrichment at the promoter region of Sox-2 in BFF cells treated with and without PD0325901, CHIR99021, and NuP0178 (3i) at day 7 and day 14. Enrichment is expressed as a ratio of immunoprecipitated DNA over input DNA. Percent of Input was calculated using the following equation: Input % = 100/2^(ΔCt [normalized ChIP]), where ΔCt [normalized ChIP] = (Ct [ChIP] - (Ct [Input] - Log2 (Input Dilution Factor)). This data is presented in Table 4.2. and Table 4.5. Time was observed to have a significant effect on H3K27me3 enrichment at the Sox-2 promoter (p = 0.04), and is illustrated above.
Discussion

The aim of this experiment was to determine what, if any, effect treatment of BFF cells with a small molecule inhibitor cocktail comprised of CHIR99021, PD0325901, and NuP0178 has on the methylation levels of H3K4, H3K9, and H3K27 on the promoters of the core pluripotency-associated genes, Oct-4, Nanog, and Sox-2. Building upon this design, we expanded our query to determine what, if any, effect the length of treatment has on the methylation status of these histone residues. Generally methylation of H3K4 is associated with transcriptional activation, whereas methylation of H3K9 and H3K27 is mainly associated with transcriptional repression. Several small molecules, affecting specific signaling pathways and/or chromatin modifications, have been shown to improve both the kinetics and efficiency of reprogramming (summarized in Table 3.1) (Zhang et al., 2012). These chemical modifiers aid in overcoming the “roadblocks” encountered during the reprogramming process by inducing the necessary epigenetic modifications needed to silence the somatic cell genome and completely reactivate the ESC genome. Chemical treatment of cells prior to reprogramming can remodel the epigenetic landscape of the somatic cell type to be more like that of ESC by removing the repressive epigenetic marks characteristic of somatic cells and relaxing chromatin structure to allow the reprogramming factors easier access to target genes. It has been suggested that the genome of less differentiated cells may be more amenable to reprogramming or require less reprogramming following the induction of pluripotency either by SCNT or another experimental strategy (Rideout et al., 2001). Currently, the overall efficiency of SCNT is between 0-3% (number of live offspring as a percentage of the number of nuclear transfer embryos) (Paterson et al., 2003). While several factors
have been identified as contributors to the inefficiency of the procedure, incomplete epigenetic reprogramming is considered the primary reason for developmental failure of SCNT embryos (Li et al., 2003). Based on this notion, we hypothesized that treatment with CHIR99021, PD0325901, and NuP0178 (3i) will remodel the epigenetic landscape of BFF cells via histone modifications so that they may ultimately be more easily reprogrammed by means such as SCNT.

To examine the effect of 3i treatment on histone methylation at the lysine 4, 9, and 27 residues in Oct-4, Nanog, and Sox-2, we utilized ChIP, a powerful and popular tool for understanding the mechanisms of gene regulation by transcription factors and modified histones. Using antibodies that recognize a specific protein or protein modification of interest, ChIP determines the relative abundance of that protein or protein modification of interest at one or more locations in the genome. ChIP is the most widely used procedure for the examination of histone modification, and it has proven to yield very valuable information on chromatin-associated processes in numerous species. Nevertheless, the technique must be optimized by each investigator in their model system specific. Optimization of cell harvesting, the cross-linking of chromatin, sonication conditions, and qRT-PCR setup and analysis of ChIP-enriched genomic DNA is very tedious, challenging, and time-consuming. Indeed, this proved to be true for this research project. We originally used enzymatic digestion, rather than sonication, to shear chromatin for ChIP because it is said to greatly simplify and streamline the ChIP protocol, as well as reduce disruption of protein/DNA complexes traditionally caused by sonication. Unfortunately, enzymatic shearing proved to be ineffective in our model system, leading us to conclude that BFF may be among those
cell types more resistant to cell lysis. We changed our ChIP protocol from enzymatic shearing to mechanical shearing of chromatin by sonication. Optimization of the amended ChIP protocol included the individual optimization of many variables including, cell harvesting technique, cell fixation time, cell lysis by dounce homogenization, sonication conditions, and qRT-PCR setup and analysis of the ChIP material. The ChIP protocol optimized for our model system and implemented in this study is described in Appendix A.

BFF cells from seven different cell lines were cultured with and without CHIR99021, PD0325901, and NuP0178 for a total of 14 days. Using the optimized ChIP protocol established for our model system, chromatin was prepared from these cultures on day 7 and day 14. The resulting chromatin was then immunoprecipitated with antibodies directed against H3K4me3, H3K9me3, and H3K27me. Following immunoprecipitation, DNA was recovered and analyzed by qRT-PCR to identify DNA loci associated with these histone modifications at the promoters of Oct-4, Nanog, and Sox-2. Enrichment of precipitated DNA associated with either H3K4me3, H3K9me3, or H3K27me3 is represented as a percentage of the input (Table 4.2 and Table 4.3). ChIP followed by qRT-PCR revealed that a great deal of variability in H3K4, H3K9, and H3K27 trimethylation of Oct-4, Nanog, and Sox-2 exists between samples. This is seen in both control and treatment cells at both time points. This variability can be attributed to several different factors, including the method used for normalizing the ChIP data.

The two most common ways to normalize data is the Fold Enrichment method and the Percent of Input method. The Fold Enrichment method of normalization assumes the level of background signal reproducible between different primer sets,
samples, and replicate experiments. Fold Enrichment is calculated by taking the ChIP signal and dividing it by the No antibody signal, representing the ChIP signal as a fold increase relative to the background signal. Conversely, the Percent of Input method of normalization takes the ChIP signal and divides it by the signal measured from the input sample. The input sample is a positive control that does not go through the immunoprecipitation process, correlating to the total available promoters in the chromatin. It is indicative for the presence and amount of chromatin used in the ChIP reaction. It is assumed that the obtained ChIP and No antibody signal levels are directly related to the amount of input chromatin (Haring et al., 2007). After thoroughly researching these two methods of normalization, we concluded that the Percent of Input method was the best method of normalization for this experiment. Because background signal levels do vary between primer sets, samples, and experiments, the assumption of the Fold Enrichment method - the level of background signal is reproducible between different primer sets - often results in a random over- or under-representation of the ChIP data (Haring et al., 2007). While we contend that the Percent of Input method is the optimal method of data normalization, it also has its drawbacks. The main disadvantage of this method is caused by differences in handling the input and ChIP samples. Because the input sample is taken so early in the preparation process, it is subjected to many opportunities for unequal handling, possibly rendering the input and ChIP samples different from one another. There is no consensus on how to normalize ChIP-qPCR data within the literature because there is no perfect method of normalization. While the Percent of Input method is the preferred method of normalization for the majority of recent studies, the inherent nature of the procedure
subjects data to potential variability, which could possibly be the reason for the variability observed in this study. Lastly, it is important to remember that enrichment of a target is not solely dependent on the quantity on the antigen associated with it. Immunoprecipitation is affected by the accessibility of that antigen in that particular chromatin environment, the affinity of the antibody and the precise conditions of the immunoprecipitation process. For this reason, the level of enrichment is always expressed as a ratio of the precipitated sequence over the input. Therefore, the absolute levels of different antigens present in the same sequence cannot be compared directly to one another.

Our previous examinations of Oct-4, Nanog, and Sox-2 transcript levels in sources of ASC, including BFF, revealed capricious expression of these transcripts between replicates (Coley, 2007). Taking this into consideration may help explain the variability in enrichment of H3K4me3, H3K9me3, and H3K27me3 at the promoter regions of these transcripts between samples. Furthermore, the molecular mechanisms determining the turnover rate of histone modifications can influence data interpretation (Clayton et al., 2006). Particular histone modifications can exist as very transient marks, including methylation of H3K4 (Morillon et al., 2005). When a particular histone modification has a high turnover rate, only a subset of the crosslinked nucleosomes will carry that modification, resulting in a lower ChIP signal than when that same modification is continuously present (Haring et al., 2007).

While the scarcity of stem cells in most tissues remains a major challenge in studying adult stem cells, several groups were able to isolate sufficient quantities of adult stem cells from tissues to conduct transcriptional and epigenetic profiling studies.
Results from the limited number of studies presently available support the notion that the chromatin states of adult stem cells are intermediate between those of pluripotent cells and terminally differentiated cells. For example, while the chromatin of adult stem cells is globally less `open' compared with that of ES cells, a common set of stemness genes, including regulators of chromatin, transcription, cell cycle and survival, is marked by H3K4me3 and is active in both HF-SCs and ES cells (Lien et al., 2011). In HSCs, H3K4me3 is more prevalent compared with differentiated progeny, and enhancers of differentiation genes are marked by monomethylation of H3K4, H3K9, and H3K27, which is likely involved in the maintenance of activation potential required for differentiation. Specifically, gene expression positively correlates with H3K4me3, H3K4me1, H3K9me1, H3K36me3, and H4K20me1 and negatively correlates with H3K9me3 and H3K27me3 (Cui et al., 2009; Chen and Dent, 2014).

It is important to note that Marks et al. (2012) demonstrated that mouse ESC grown in media containing CHIR99021 and PD0325901 exhibit similar H3K4me3 profiles, but substantially reduced prevalence of H3K27me3 at promoters, many less bivalent domains, and lower- rather than higher- expression of lineage-specific genes, compared to those cells grown in culture-containing medium. Therefore, a large proportion of bivalent domains in ESC cultured in serum are due to the acquisition of H3K27me3 at promoters (Marks et al., 2012; Chen and Dent, 2014).

Transcription factors preferentially bind to `open' chromatin. Thus, epigenetic mechanisms may set the stage for lineage-specific transcription factors by creating and maintaining a permissive chromatin environment. Indeed, an emerging theme from recent studies is that epigenetic pre-patterning occurs before cell fate decisions. In one
study, Szutorisz and colleagues (2005) differentiated mouse ES cells toward the B-cell lineage and investigated the epigenetic regulation of gene expression. They found that a \textit{cis}-acting element in the immunoglobulin lambda-like polypeptide 1 (\textit{Igll1}; also known as \textit{λ}5–pre-B lymphocyte gene 1 (\textit{VpreB1}) locus is marked by histone H3ac and H3K4me2 at a discrete site in undifferentiated ES cells. The marked region expands during differentiation and becomes a localized center for transcription factors and RNA polymerase II recruitment before full activation of the \textit{Igll1} and \textit{VpreB1} genes at the pre-B cell stage (Szutorisz et al., 2005) Similarly, Xu et al. (2011) showed that the liver and pancreas regulatory elements have distinct chromatin patterns in undifferentiated endoderm cells. When the cells differentiate into hepatoblasts, acetylation of H3K9 and H3K14 promote expression of hepatic genes, and H3K27me3 appears to repress the expression of pancreatic genes (Xu et al., 2011). The concept of transcriptional priming by chromatin changes is reinforced by recent studies of higher-order chromatin structure during induced `dedifferentiation'. Circular chromosome conformation capture with high-throughput sequencing (4C-seq) reveals that, during somatic cell reprogramming into iPSC, the establishment of long-range interchromosomal interactions with the Oct-4 and Nanog loci precedes transcriptional activation of these genes (Apostolou et al., 2013; Wei et al., 2013; Chen and Dent, 2014). BFF treated with CHIR99021, PD0325901, and NuP0178 may ultimately be more easily reprogrammed by means such as SCNT than other somatic cells that have not undergone pre-treatment with small molecules because they may “prime” BFF cells for nuclear reprogramming by inducing epigenetic changes permissible to nuclear reprogramming. While we cannot exclude that the significant effects observed in this
experiment are simply the result of randomness, we cannot exclude that they may in fact be due to the effects of treatment with these three small molecules priming BFF cells for nuclear reprogramming.
CHAPTER V
THE EFFECT OF CHIR99021 AND PD325901 TREATMENT ON PLURIPOTENT TRANSCRIPT EXPRESSION AND HISTONE ACETYLATION IN BOVINE FETAL FIBROBLAST CELLS

Introduction

Attempts to derive iPSC from livestock species have been met with limited success. Although there have been reports of reprogramming somatic cells towards pluripotency in livestock species, such as the pig, cow, and buffalo (Huang et al., 2011; West et al., 2011; Deng et al., 2012), almost all of the reported livestock iPSC were not capable of forming germ line chimeras. Differences in ESC and iPSC properties between the mouse and the human have been well documented, as these have been studied the most. Similarly, studies in other mammalian species, include the cow, pig, and rat, for example, have shown clear differences in preimplantation development, embryonic transcript expression patterns, and the conditions required to maintain proliferative and undifferentiated cells in culture (Telugu et al., 2010). Even in the mouse and human, gene expression networks that support pluripotency and proliferation are still not completely understood, although it is clear that two or more signal transduction pathways, probably acting in parallel must exist to account for the different cell types. While it is likely that some version of these pathways also support ESC identity in ungulate systems, it is thought that the cell signaling pathways underlying pluripotency and nuclear reprogramming may differ across species; therefore, the effectiveness of certain reprogramming techniques and enhancement strategies may vary cross species (Telugu et al., 2010). Because the correct expression profile of pluripotency-related genes is critical for the derivation and
maintenance of pluripotent cells, it is necessary to identify the specific molecular machinery regulating pluripotency in the bovine in order to create iPSC from this species.

A number of small molecule compounds have been reported to facilitate and/or enhance iPSC production in a variety of species. CHIR99021 and PD325901 (2i) has been widely used to enhance to reprogramming in the mouse and human (Li et al., 2009; Lin et al., 2009; Hanna et al., 2010; Zhou et al., 2010; Li et al., 2011; Marks et al., 2012). For this reason, 2i has been adopted into reprogramming systems in other species as well. However, its effectiveness in mammalian species other than the mouse and human has been brought into question as some studies in the pig have shown reduced pluripotent gene expression in 2i-treated cells (Telugu et al., 2011; Gao et al., 2013; Petkov et al., 2014). It is possible that the molecular network responsible for establishing and maintaining pluripotency in the pig may differ from that in the mouse and human. This could similarly be the case for other mammalian species, including bovine. Therefore, the effectiveness of 2i treatment on bovine somatic cell types needs to be assessed to determine if it is suitable for the pre-treatment of donor cells for SCNT. Because less-differentiated cell types are more readily reprogrammed than their terminally differentiated counterpart, it is important to ascertain if 2i maximizes epigenetic priming for successful reprogramming.

An important aspect of using reprogrammed cells as donor cells for SCNT is the ability to treat and expand cells to a sufficient quantity. Typically somatic cells have a limited proliferative capacity; therefore, it is important genetic modifications and subsequent preparation for SCNT be accomplished before the cells enter senescence.
It has previously been estimated that ~45 population doubling (PD) are required to transfet, select, expand, and prepare cells for SCNT. Therefore, the longevity of donor cells in culture is a key parameter in the development of ideal donor cells for SCNT (Denning et al., 2001).

Materials and Methods

Experiment 1 Experimental Design

In the present study, we assessed the individual and combinatorial effects of CHIR99021 and PD0325901 treatment on Oct-4, Nanog, and Sox-2 relative expression in BFF cells over time. BFF cells were allowed to expand in culture before being split evenly into seven groups and treated as follows: (1) 3 µM CHIR99021 prepared from 10 mM stock solution in DMSO (Stemgent, Lexington, MA, Cat.no. 04-0004-02); (2) 0.5 µM PD0325901 prepared from 10 mM stock solution in DMSO (Stemgent, Lexington, MA, Cat.no. 04-0006-02); (3) 3 µM CHIR99021 + 0.5 µM PD0325901; (4) 1.5 µM CHIR99021 + 0.25 µM PD0325901; (5) 6 µM CHIR99021 + 1 µM PD0325901; (6) complete culture medium + 0.053% DMSO; (7) 1 µM TSA prepared from 5 mM stock solution in DMSO (Sigma-Aldrich, T1952). BFF cells were treated for a total of 14 days, and on days 7 and 14 mRNA was isolated from all cultures for gene expression analysis. qRT-PCR was performed to measure transcript levels for Oct-4, Nanog, and Sox-2 in all BFF cell cultures.

Experiment 2 Experimental Design

To explore the effects of CHIR99021 and PD0325901 treatment at varying concentrations as mentioned above on global histone acetylation, flow cytometry was
performed on cells harvested from the seven treatment groups on days 7 and 14 of culture. Cells were fixed, permeabilized, and incubated with anti-acetyl-histone H3 antibody to determine the total level of H3 acetylation (H3ac) using flow cytometry.

Experiment 3 Experimental Design

As previously mentioned, we have observed adverse side effects following treatment with 3i (CHIR99021, PD0325901, NuP0178) resulting in reduced cell survival. To explore the potential culprit causing this, we studied growth characteristics of cells cultured under the seven conditions mentioned above and calculated the population doublings after 7 and 14 days of treatment.

Establishment of Cell Lines and Maintenance

Target cell lines were obtained from previously isolated primary cultures. Primary cultures of fibroblast cells were established from bovine fetuses approximately 50 days old according to protocol (Giraldo et al., 2009). These Bovine Fetal Fibroblast (BFF) cells were cultured in complete culture medium composed of Dulbecco’s Modified Eagle Medium (DMEM) with high glucose, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (P/S). Once cells reached 80% confluency, cultures were passaged by releasing cells with trypsin (0.25%). BFF cells were then resuspended in DMEM supplemented with 10% bovine calf serum (CS) and 10% dimethyl sulfoxide (DMSO) for cryopreservation. Cryovials containing approximately 1,000,000 BFF cells suspended in solution were cooled at 1°C/min until reaching -80°C before storage in liquid nitrogen.
BFF cells were thawed as needed at room temperature for 30 sec, followed by submersion in a 38°C water bath. Cells were then washed in complete culture medium and seeded at a density of 0.3 x 10^6 into 6 well plates and cultured under 5% CO_2 and 90% humidity at 37°C.

Treatment with Small Molecules

Once BFF cells expanded to a sufficient quantity, cells were split into 6-well tissue culture dishes at a seeding density of 0.3 x 10^6 and treated under the appropriate conditions defined for the seven treatment groups. All media was prepared with complete culture medium (DMEM + 10% FBS + 1% P/S) and 10 mM stock solutions of the small molecule inhibitors CHIR 99021 (Stemgent, Lexington, MA, Cat. No. 04-0004-02) and PD0325901 (Stemgent, Lexington, MA, Cat. No. 04-0006-02). Because the stock solutions of the small molecule inhibitors are reconstituted in DMSO, which can potentially be toxic to cells, DMSO was added to complete culture medium (DMEM + 10% FBS + 1% P/S + 0.053% DMSO) to control for this possibility. This concentration of DMSO used was determined based on the concentrations of CHIR99021 and PD0325901 in the other media preparations. Therefore, all cells were exposed to DMSO at approximately the same concentration. The treatments are as follows: (1) 3 µM CHIR99021 (CHIR); (2) 0.5 µM PD0325901 (PD); (3) 3 µM CHIR99021 + 0.5 µM PD0325901 (2i); (4) 1.5 µM CHIR9901 + 0.25 µM PD0325901 (2i*); (5) 6 µM CHIR99021 + 1 µM PD0325901(2i**); (6) 1 µM Trichostatin A (TSA); (7) DMEM + 10% FBS + 1% P/S + 0.053% DMSO (control). Cells were then placed in 37°C incubator with 5% CO_2 and 90% humidity. Media was changed every 1-2 days.
Cell Growth Characteristics and Population Doublings

On days 7 and 14 cells were harvested for downstream applications, including qRT-PCR and flow cytometry. Cells were trypsinized and washed in 1 ml of Dulbecco’s Phosphate Buffered Solution (DPBS) with Ca\(^{+2}\) and Mg\(^{+2}\). Cells were counted using a hemacytometer to determine concentration. The average concentration of cells from each culture group was calculated and used to determine population doublings (PDs). The following formula was used to calculate PDs: \(PD = 3.32 \times (\log X_e - \log X_b)\), where \(X_b\) is the cell number at the beginning of the incubation time and \(X_e\) is the cell number at the end of the incubation time (Patterson, 1979). Pictures of the cells in culture were also taken on days 7 and 14 to compare growth characteristics of cells between treatments and over time.

Isolation of mRNA

On days 7 and 14, both treatment and control cells were harvested by trypsinization and washed in 1 ml of Dulbecco’s Phosphate Buffered Solution (DPBS) with Ca\(^{+2}\) and Mg\(^{+2}\). Cells were counted using a hemacytometer to determine concentration. Cells were pelleted by centrifugation and stored at -80° C to until required for RNA purification. Because the concentration of cells harvested from cultures was relatively low, RNA isolation was performed using RNeasy® Mini Kit (Qiagen, Cat No. 74106). Once removed from -80°C, cell pellets were loosened by flicking the tube, and either 350 µl (<=5 \times 10^6) or 600 µl (5 \times 10^6 – 1 \times 10^7) of Buffer RLT was added to the tube. Cell lysates were homogenized by passing the lysate through a 21 gauge needle 5-8 times. One volume 70% ethanol was added to the lysate before
transferring 700 µl of the cell suspension to an RNeasy spin column placed in a collection tube. Samples were centrifuged for 15 s at 8000 x g and flow-through discarded. 700 µl of Buffer RW1 was added to the column and centrifuged again. Next, the column was washed twice in 500 µl Buffer RPE. RNA was eluted by adding 30 µl RNase-free H2O to the column followed by a 1 min spin at 8000 x g.

Quantitative Real-Time PCR

Our laboratory has previously analyzed BFF cells for Oct-4, Nanog, and Sox-2 expression, thus we have validated the specificity of primers for PAP, Oct-4, Nanog, and Sox-2 (Coley, 2010). These primers were designed from bovine gene sequences using the Beacon Designer 4.0 (PREMIER Biosoft International) (Table 5.1), and were diluted to 10 mM concentration. We extended gene expression analysis to include CTNNB1 and EGF in order to assess the specificity of CHIR99021 and PD0325901, respectively. To explore the high incidence of cell death observed in BFF cells treated with small molecule inhibitors, BFF cells were also analyzed for the expression of p53, which activates the senescent pathway (Sharpless and DePinho, 2002). In addition to PAP, β-actin and GAPDH were used as housekeeping genes for standardization. The qRT-PCR primers used for amplification of these genes have previously been validated by others (Table 5.1). Specificity of the primer sequences was confirmed by amplification in calibrator sample. RNA was amplified using SsoFast™ EvaGreen supermix (Bio- Rad Laboratories, Inc., Hercules, CA, USA). The total 20 µl real time
<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Primers</th>
<th>Annealing Temp</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td>XM_002696890.2</td>
<td>Sense AGTTGGGCACTTTTGAAGACC</td>
<td>64°</td>
<td>(Takatsu et al., 2015)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense AGGACCACCTCACAGTTGTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward TGCCCATCTATGAGGGGTACG</td>
<td>60°</td>
<td>(Lu et al., 2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse CGCTCCGTGAGGATCTTCATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTNNB1</td>
<td>NM_001076141.1</td>
<td>Sense CTCAGTCCTCTGCCCATACTA</td>
<td>50°</td>
<td>(Matwee et al., 2000; Favetta et al., 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense GGATCCAGGATAAGGTGAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P53</td>
<td>U74486</td>
<td>Sense CTCAGTCCTCTGCCCATACTA</td>
<td>50°</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense GGATCCAGGATAAGGTGAGC</td>
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<tr>
<td></td>
<td></td>
<td>Sense CGTGACATTAAGGAGAAGCTGTCG</td>
<td>60°</td>
<td>(Favetta et al., 2004)</td>
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<tr>
<td></td>
<td></td>
<td>Antisense CTCAGGAGGAGGAATGATCTTGTG</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Sense CTCCTATGGACCTTACATCGTCTA</td>
<td>60°</td>
<td>Adams, 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense TGGAAATGATGATGCGCTTCCATTG</td>
<td></td>
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</tr>
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</table>
PCR mix consisted of 2 μl of cDNA, 10 μl of SsoFast™ EvaGreen Supermix, 6 μl of nuclease-free water, and 1 μl of forward and reverse primer pairs (10 mM) for each gene. All qRT-PCR was performed using Bio-Rad CFX Connect™ Real-Time PCR Detection System. The PCR program used for the amplification of all primers consisted of a denaturing cycle of 30 sec at 95°C; 40 cycles of PCR at 95°C for 5 sec and 50-60°C (primer-specific annealing temperature (Table 5.1) 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 4°C.

Data was quantified using a modified ∆∆Ct method described by (Hellemans et al., 2007). Values are reported as relative transcription or the n-fold difference relative to a calibrator. A mixture of cDNA from BFF cells at multiple passages was used as a calibrator for all of the target genes. The geometric mean of PAP, GAPDH, and β-actin was used to normalize data. Therefore, transcript abundance is calculated relative to each reference gene and averaged using the geometric mean.

Flow Cytometry

Flow cytometry was performed using a protocol modified from (Habib et al., 1999). On days 7 and 14, both treatment and control cells were harvested by trypsinization and washed in 1 ml of PBST-BSA (DPBS without Ca+2 and Mg+2 + 1% BSA + 0.1% Tween20). Cells were counted using a hemacytometer to determine concentration of cells in each culture. The cell suspension was then divided evenly between two Falcon® 5 mL polystyrene tubes (Cat. No. 352054) to prepare a negative autofluorescence control for each sample. Dissociated cells were then resuspended in
2 mL 0.25% paraformaldehyde in DPBS, and allowed to incubate for 10 min at RT for cell fixation. Cells were centrifuged at 400 x g for 5 min and resuspended in 200 µl of PBS containing 1% BSA (Fraction V). To detect intracellular molecules, cells were permeabilized by slowly adding 1.8 mL of ice-cold methanol to the cell suspension. Cells were then incubated for 30 min at -20° C. Cells were centrifuged at 400 x g for 5 min at 4°C, methanol discarded, and washed in PBS containing 1% BSA. Cells were incubated in Anti-acetyl-Histone H3, Alexa Fluor® 488 Conjugate (Millipore, Temecula, CA, Cat. No. 06-599-AF488) antibody diluted 1:100 in PBS containing 1% BSA for 1 h at 4°C. Equal volume PBS containing 1% BSA was added to the negative autofluorescence control tubes. Afterwards, cells were resuspended in 2 mL PBS containing 1% BSA, centrifuged at 400 x g for 5 min, and the supernatant discarded. Lastly, cells were resuspended in 300 µl 0.25% paraformaldehyde in DPBS to preserve samples for next-day analysis.

Flow cytometric analysis was used to quantify global levels of H3ac in cells CHIR99021 and PD0325901-independently and at varying concentrations in combination over time. Samples were assayed on a BD Biosciences FACS Calibur™ (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Labeled cells were excited at 488 nm with a 15-mW argon laser to determine the relative level of acetylated histone H3 measured by fluorescence of cells labeled with in Anti-acetyl-Histone H3, Alexa Fluor® 488 Conjugate antibody. Data generated in flow cytometry was analyzed by the BD FACStation™ data management system, and the percentage of cells manifesting specific fluorescence for the Anti-acetyl-Histone H3, Alexa Fluor® 488 Conjugate antibody was calculated. A negative autofluorescence control for each sample was
used to identify forward and side scatter characteristics of the cell population, and subsequently used to set PMT voltage and negative gates.

Statistical Analysis

In experiment 1, a completely random design with repeated measures was employed to assess any effect treatment with CHIR99021 and PD035901- independently and in varying combinations- had on target gene expression in BFF cells over time. The PROC MIXED with repeated measures analysis with autoregression of order one covariance was used in SAS statistical software to determine any statistical differences in relative gene expression between treatment and control groups over time.

In experiment 2, ANOVA to determine variance in relative levels of H3ac between control BFF cells and BFF cells treated with CHIR99021 and PD0325901- independently and at varying concentrations in combination over time.

Results

Gene Expression Analysis

The values corresponding to the relative expression of Oct-4, Nanog, Sox-2, CTNNB1, EGF, and p53 in BFF cells treated in the seven culture systems tested on day 7 and day 14 are represented in figures 5.1, 5.2, 5.3, and 5.4. To ensure that CHIR99021 and PD0325901 are active in bovine cells under the experimental conditions, β-catenin (CTNNB1) and epidermal growth factor (EGF) were used as positive control genes.
**Relative Gene Expression in CHIR99021 and PD0325901 Culture Systems at Day 7**

**Figure 5.1.** Relative expression of Oct-4, Nanog, and Sox-2 in BFF cells treated in the following culture systems for 7 days: (1) 3 µM CHIR99021 (CHIR); (2) 0.5 µM PD0325901 (PD); (3) 3 µM CHIR99021 + 0.5 µM PD0325901 (2i); (4) 1.5 µM CHIR9901 + 0.25 µM PD0325901 (2i*); (5) 6 µM CHIR99021 + 1 µM PD0325901(2i**); (6) 1 µM Trichostatin A (TSA); (7) DMEM + 10% FBS + 1% P/S + 0.053% DMSO (control). Experimental procedures were replicated in four different BFF cell lines. Target gene abundance levels were normalized using the geometric mean of PAP, GAPDH, and β-actin. Statistical significance determined as p = 0.05.
Figure 5.2. Relative expression of CTNNB1, EGF, and p53 in BFF cells treated in the following culture systems for 7 days: (1) 3 µM CHIR99021 (CHIR); (2) 0.5 µM PD0325901 (PD); (3) 3 µM CHIR99021 + 0.5 µM PD0325901 (2i); (4) 1.5 µM CHIR9901 + 0.25 µM PD0325901 (2i*); (5) 6 µM CHIR99021 + 1 µM PD0325901 (2i**); (6) 1 µM Trichostatin A (TSA); (7) DMEM + 10% FBS + 1% P/S + 0.053% DMSO (control). Experimental procedures were replicated in four different BFF cell lines. Target gene abundance levels were normalized using the geometric mean of PAP, GAPDH, and β-actin. Statistical significance determined as p = 0.05 and indicated by *. 
Figure 5.3. Relative expression of Oct-4, Nanog, and Sox-2 in BFF cells treated in the following culture systems for 14 days: (1) 3 µM CHIR99021 (CHIR); (2) 0.5 µM PD0325901 (PD); (3) 3 µM CHIR99021 + 0.5 µM PD0325901 (2i); (4) 1.5 µM CHIR9901 + 0.25 µM PD0325901 (2i*); (5) 6 µM CHIR99021 + 1 µM PD0325901 (2i**); (6) 1 µM Trichostatin A (TSA); (7) DMEM + 10% FBS + 1% P/S + 0.053% DMSO (control). Experimental procedures were replicated in four different BFF cell lines. Target gene abundance levels were normalized using the geometric mean of PAP, GAPDH, and β-actin. Statistical significance determined as $p = 0.05$. 
Figure 5.4. Relative expression of CTNNB1, EGF, and p53 in BFF cells treated in the following culture systems for 14 days: (1) 3 µM CHIR99021 (CHIR); (2) 0.5 µM PD0325901 (PD); (3) 3 µM CHIR99021 + 0.5 µM PD0325901 (2i); (4) 1.5 µM CHIR9901 + 0.25 µM PD0325901 (2i*); (5) 6 µM CHIR99021 + 1 µM PD0325901 (2i**); (6) 1 µM Trichostatin A (TSA); (7) DMEM + 10% FBS + 1% P/S + 0.053% DMSO (control). Experimental procedures were replicated in four different BFF cell lines. Target gene abundance levels were normalized using the geometric mean of PAP, GAPDH, and β-actin. Statistical significance determined as p = 0.05 and indicated by *.
CHIR99021 inhibits GSK3 signaling, thus activating Wnt signaling. CTNNB1 is a major downstream component of the Wnt signaling pathway, therefore, its expression is interpreted as functional CHIR99021 in our system. EGF was used as a positive control gene for specificity of PD0325901, a MEK/ERK signaling pathway inhibitor. Consistent with the results of 3i treatment, statistical analysis comparing Oct-4, Nanog, and Sox-2 expression levels in cells treated with CHIR99021 and PD0325901 - independently and in combination at various concentrations - revealed no significance difference in gene expression between groups at either day 7 or day 14. Conversely, a significant difference in p53 expression was observed in 2i** media. Overall, p53 expression increased in culture systems containing CHIR99021 and PD0325901, though not all were of statistical significance.

The components of 2i+ culture medium involve several signaling pathways regulating self-renewal and pluripotency. To evaluate the function of these pathways in bovine cell types, BFF cells were treated as follows: (1) 3 µM CHIR99021 (CHIR); (2) 0.5 µM PD0325901 (PD); (3) 3 µM CHIR99021 + 0.5 µM PD0325901 (2i); (4) 1.5 µM CHIR9901 + 0.25 µM PD0325901 (2i*); (5) 6 µM CHIR99021 + 1 µM PD0325901(2i**); (6) 1 µM Trichostatin A (TSA); (7) DMEM + 10% FBS + 1% P/S + 0.053% DMSO (control). Pictures of cells from each treatment group on days 7 and 14 were captured to illustrate the effects of small molecule treatment on cell growth (Figure 5.5 and 5.6).
Figure 5.5. BFF cells cultured in (A) 3 μM CHIR99021 (CHIR); (B) 0.5 μM PD0325901 (PD); (C) 3 μM CHIR99021 + 0.5 μM PD0325901 (2i); (D) 1.5 μM CHIR9901 + 0.25 μM PD0325901 (2i*); (E) 6 μM CHIR99021 + 1 μM PD0325901(2i**); (F) DMEM + 10% FBS + 1% P/S + 0.053% DMSO; (G) 1 μM Trichostatin A (TSA) for 7 days
Figure 5.6. BFF cells cultured in (A) 3 µM CHIR99021 (CHIR); (B) 0.5 µM PD0325901 (PD); (C) 3 µM CHIR99021 + 0.5 µM PD0325901 (2i); (D) 1.5 µM CHIR9901 + 0.25 µM PD0325901 (2i*); (E) 6 µM CHIR99021 + 1 µM PD0325901(2i**); (F) DMEM + 10% FBS + 1% P/S + 0.053% DMSO; (G) 1 µM Trichostatin A (TSA); for 14 days.
Noticeable differences in cell morphology and growth can be seen between culture groups over time. Notably, cells treated with PD, 2i, and 2i** exhibit reduced cell growth in culture compared to the other groups. This observation correlates to the population doublings calculated at day 7 and day 14, which show a reduction in cell concentration (Table 5.2). All cells were seeding at a density of $0.3 \times 10^6$ into 6 well plates. On days 7 and 14 cells were harvested for downstream applications, and the cells were counted. The population doublings for DMSO treated cells were consistent with BFF cell growth in culture (Giraldo et al., 2009). The counts at the time of harvest for PD, 2i, 2i*, and 2i** treated cells were all either reduced or approximately the same as the seeding density. CHIR treated populations increased to approximately $\sim 0.5 \times 10^6$ cells at the time of harvest, but still lower than what is anticipated for BFF cells under normal culture conditions. This illustrates the difficulty in growing cells to a sufficient quantity for subsequent use as donor cells for NT in the presence of these small molecule inhibitors.

Flow Cytometric Analysis of Histone H3 Acetylation

Flow cytometric analysis was performed on cell populations treated under seven culture conditions (CHIR; PD; 2i; 2i*; 2i**; TSA, DMSO) for 7 days and 14 days to determine global H3ac levels (Figure 5.7 and 5.8). The experiment was performed on four BFF cell lines, and the mean percent of positive values generated for H3ac is reported in Table 5.3. Treatment with Trichostatin A (TSA) was used as a positive control for our experimental designs. TSA is a member of the subgroup of HDAC inhibitors that induce tubulin hyperacetylation. Therefore, an increased amount of H3ac
Table 5.2. Population doubling of BFF cells after 7 and 14 days of treatment in culture media

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>Day 7</th>
<th>Day14</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHIR</td>
<td>0.83</td>
<td>0.56</td>
</tr>
<tr>
<td>PD</td>
<td>-0.60</td>
<td>-0.60</td>
</tr>
<tr>
<td>2i</td>
<td>0.40</td>
<td>0.20</td>
</tr>
<tr>
<td>2i*</td>
<td>-0.27</td>
<td>-0.17</td>
</tr>
<tr>
<td>2i**</td>
<td>-0.90</td>
<td>-1.26</td>
</tr>
<tr>
<td>DMSO</td>
<td>1.53</td>
<td>1.94</td>
</tr>
<tr>
<td>TSA</td>
<td>0.94</td>
<td>0.78</td>
</tr>
</tbody>
</table>
Figure 5.7. Histograms representing the percentage of positive cells for Histone H3 Acetylation in CHIR99021 and PD0325901 in BFF cells cultured in (A) 3 µM CHIR99021 (CHIR); (B) 0.5 µM PD0325901 (PD); (C) 3 µM CHIR99021 + 0.5 µM PD0325901 (2i); (D) 1.5 µM CHIR9901 + 0.25 µM PD0325901 (2i*); (E) 6 µM CHIR99021 + 1 µM PD0325901(2i**); (F) DMEM + 10% FBS + 1% P/S + 0.053% DMSO; (G) 1 µM Trichostatin A (TSA) after 7 days.
Figure 5.8. Histograms representing the percentage of positive cells for Histone H3 Acetylation in CHIR99021 and PD0325901 in BFF cells cultured in (A) 3 µM CHIR99021 (CHIR); (B) 0.5 µM PD0325901 (PD); (C) 3 µM CHIR99021 + 0.5 µM PD0325901 (2i); (D) 1.5 µM CHIR9901 + 0.25 µM PD0325901 (2i*); (E) 6 µM CHIR99021 + 1 µM PD0325901(2i**); (F) DMEM + 10% FBS + 1% P/S + 0.053% DMSO; (G) 1 µM Trichostatin A (TSA) after 14 days.
in cells cultured in TSA demonstrates that the flow cytometric techniques used in this experiment are sufficiently specific and sensitive to detect changes in levels of acetylated histone H3 compared to those induced by this chemical.

Table 5.3. Percentage of Positive Cells for Histone H3 Acetylation in CHIR99021 and PD0325901 Culture Systems at Days 7 and 14

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>Mean % Positive Cells D7</th>
<th>Mean % Positive Cells D14</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHIR</td>
<td>77.0%</td>
<td>93.5%</td>
</tr>
<tr>
<td>PD</td>
<td>80.7%</td>
<td>81.4%</td>
</tr>
<tr>
<td>2i</td>
<td>77.1%</td>
<td>80.4%</td>
</tr>
<tr>
<td>2i*</td>
<td>72.7%</td>
<td>78.6%</td>
</tr>
<tr>
<td>2i**</td>
<td>72.9%</td>
<td>83.3%</td>
</tr>
<tr>
<td>TSA</td>
<td>78.1%</td>
<td>91.9%</td>
</tr>
<tr>
<td>DMSO</td>
<td>4.0%</td>
<td>3.86%</td>
</tr>
</tbody>
</table>
Discussion

Senescence represents an reversible state at the G1 phase of the cell cycle that is induced by replicative exhaustion or in response to stress on the cell. Several factors may contribute to the induction of apoptosis in cells reprogrammed towards pluripotency, including the level of reactive oxygen species (ROS). High ROS levels promote the modification of individual nucleotide bases, the formation of single and double strand breaks, and leads to telomere shortening, all of which results in the activation of p53 (Sharpless and DePinho, 2002; Favetta et al., 2004; Hong et al., 2009). Studies in PD0325901 have shown that PD0325901 significantly inhibited the growth of PTC cells harboring a BRAF mutation at very low concentration (10 nmol/L). They determine the mechanism of growth inhibition to be caspase 3, indicative of apoptosis (Henderson et al., 2010).

Recent studies suggest that p53 recognizes short and damaged telomeres, activating the senescent pathway. Furthermore, loss of p53 function reduces the signs of aging irrespectively of telomere length, suggesting the role for p53 rather than telomeres in the induction of cellular senescence. In mouse and human diploid fibroblast, downregulation of p53 expression extends their in vitro life span and leads to rapid cell cycle re-entry. In vitro studies in mice also indicate that senescence is caused by the activation of a p53-dependent cell cycle checkpoint that prevents cell proliferation. The p53 tumor suppressor protein is a potent transcription factor that can transactivate genes involved in both growth arrest and apoptosis (Helleman et al., 2011; Henderson et al., 2010). It is important to remember that the usefulness of quiescent cells has been attributed to their reduced transcriptional activity and chromatin
modifications that are associated with cells in G0, which may enhance their epigenetic plasticity (Armstrong et al., 2006).

These findings and ours are in support to those of Tsutsui et al (2011). They concluded that the key effect of CHIR99021 therefore does not involve the induction of Nanog. Because Nanog-overexpressing ES cells are independently blocked in differentiation, this result further suggests that the contribution of GSK3 inhibition extends beyond limiting differentiation. To probe this further, they evaluated whether CHIR99021 could rescue ESC subjected to a more profound blockade of phospho-ERK. A higher dose of PD0325901 (2 or 3 μM) almost entirely eliminates phospho-ERK and causes growth arrest and cell death. The addition of CHIR99021 restores viability and allows efficient expansion of undifferentiated ES cells in the near absence of ERK signaling. Therefore, as phospho-ERK is diminished, down modulation of GSK3 becomes increasingly crucial to maintain metabolic activity, biosynthetic capacity and overall viability. Tsutsui et al., 2011 concluded that the pivotal contribution of GSK3 inhibition is to restore full growth and viability. This may be achieved by balancing the loss of ERK input into basic cellular processes. Additionally, they did not detect any apoptotic activity with CHIR999021, consistent with our findings as well.

Further investigation into the actual cause of the observed cell growth inhibition be it apoptosis or quiescence should be investigated. More sensitive techniques that probe the actual cause of this observation, such as click-iT® TUNEL Alexa Fluor Imaging Assay (Cat. No. C110246; Invitrogen) used by Huang et al., 2011 to detect apoptosis in experimental cell lines. Similarly, Huang and colleagues (2011) used the RTCA-SP CELLigence system (Roche) to better characterize the response of
bovine cells to comparable murine and human cells. Notably, they detected a significant difference in proliferation, viability, morphology, and adhesion. By pinpointing whether these cells are entering quiescence or apoptosis as a result of treatment, as well as, species-specific response to treatment, researchers should be able to manipulate their reprogramming mechanism to induce a stably reprogrammed cell line for use in SCNT procedures specific for the species of interest. It should also be noted that while the increase in p53 expression seems contradictory to the increase in H3ac, it has been well noted that quiescent cells are easier to reprogram (Wells et al., 2013), suggesting that this data actually work in support of one another. It is important to note that very new studies suggest a strong genetic correlation to reprogramming ability (Atkinson et al., 2018). This is in support of the findings of this study, suggesting that the bovine may indeed be different than its human and murine counterpart, which may ultimately affect its ability to reprogram to a sufficient quanta of cells suitable for downstream applications such as SCNT. While it appears that the bovine is reliant on the same signaling pathways as the mouse and human for nuclear reprogramming, the mechanism of action in doing so may not be as effective as it is in their human and murine counterparts. Alternatives to PD0325901 should be explored to see their effectiveness in the bovine.
CHAPTER VI
SUMMARY AND CONCLUSIONS

In 2006, Takahashi and Yamanaka reported the generation of a population of cells similar to ESC by transfecting mouse tail fibroblast cells with four transcription factor-encoding genes. These cells, termed induced pluripotent stem cells (iPSC), are believed to be immensely beneficial in the study and treatment of disease as the direct reprogramming of somatic cells provides an opportunity to generate patient- or disease-specific pluripotent stem cells (Nakagawa et al., 2008). The supposed potential of iPSC lies in the fact that these cells are morphologically similar to pluripotent ESC and, most importantly, demonstrate key characteristics of pluripotent ESC, including expressing stem cell markers, forming teratomas containing cells of all three germ layers, and contributing to multiple cell lineages (Takahashi and Yamanaka, 2006). This landmark discovery has opened a new frontier in the field of regenerative medicine because, for the first time, a realistic way of generating sufficient numbers of patient-specific pluripotent stem cells exists (Amabile and Meissner, 2009). Furthermore, this method of generating iPSC has provided researchers with a new and unique tool to study mammalian development and the mechanisms underlying nuclear reprogramming. The breakthrough discovery that lineage-restricted somatic cells can be reprogrammed to a pluripotent state through the ectopic expression of defined transcription factors represents the culmination of over 50 years of research, and there is no sign of research slowing down anytime soon.

Considering the many advantages of a chemical approach to nuclear reprogramming, we first assessed the effect of pre-treatment with small molecule
inhibitors on the expression of Oct-4, Nanog, and Sox-2 in BFF cells. The small molecule inhibitors, CHIR99021 and PD325901, have been widely used as tools to facilitate the generation and maintenance of iPSC (Li et al., 2009; Lin et al., 2009; Hanna et al., 2010; Zhou et al., 2010). In these cases, CHIR99021 and PD325901 aid in overcoming the “roadblocks” encountered during the reprogramming process by inducing the necessary epigenetic modifications needed to silence the somatic cell genome and completely reactivate the ESC genome. We have previously shown that some sources of ASC, including BFF, express transcripts for the key pluripotency genes, Oct-4, Nanog, and Sox-2 (Coley, 2007). While these cell types’ differentiating and self-renewing capabilities are far less robust than that of ESC, we reasoned that the same system involved in promoting the pluripotent state in mESC and hESC would similarly work to maintain, or possibly enhance, the less differentiated state characteristic of BFF cells. While there was no significant increase in Oct-4, Nanog, or Sox-2 relative gene expression detected between control and treatment samples at either day 7 or day 14, it is important to note that there was no significant decrease in the relative expression of these transcripts over time either. Typically, pluripotent gene expression in ASC decreases as passage number increases (Tsai and Hung, 2012), yet the extended culture period and consequential passaging required to maintain cells in culture for an extended period of time did not result in a significant decrease in Oct-4, Nanog, or Sox-2 expression.

Expanding on our study and the evidence that small molecule compounds that target chromatin modifications and/or specific signaling pathways have proven to be effective at overcoming reprogramming hurdles, we next sought to assess the effects of
pre-treatment with the small molecules CHIR99021, PD325901, and NuP0178 on the methylation status of H3K4, H3K9, and H3K27 at the promoter regions of the key pluripotency-associated genes, Oct-4, Nanog, and Sox-2. To examine the effect of 3i treatment on histone methylation at the lysine 4, 9, and 27 residues in Oct-4, Nanog, and Sox-2, we utilized ChIP, a powerful and popular tool for understanding the mechanisms of gene regulation by transcription factors and modified histones. Using antibodies that recognize a specific protein or protein modification of interest, ChIP determines the relative abundance of that protein or protein modification of interest at one or more locations in the genome. ChIP is the most widely used procedure for the examination of histone modification, and it has proven to yield very valuable information on chromatin-associated processes in numerous species. Nevertheless, the technique must be optimized by each investigator in their model system specific. Optimization of cell harvesting, the cross-linking of chromatin, sonication conditions, and qRT-PCR setup and analysis of ChIP-enriched genomic DNA is very tedious, challenging, and time-consuming. Indeed, this proved to be true for this research project. Nevertheless, our laboratory has successfully created a working protocol for performing ChIP on BFF cells subjected to pre-treatment with the small molecule inhibitors, CHIR99021, PD325901, and NuP0178.

Perhaps the greatest hurdle presented in this research project, and consequently the greatest limitation of widespread implementation of the treatment with this combination of small molecules, is that many cells do not survive treatment. Maintaining BFF cells treated with 3i+ media for the duration of 14 days was extremely hard to do. Because of the extraordinarily high rate of cell death attributed to chemical
treatment, it was extremely difficult to harvest enough cells and subsequent chromatin required to perform ChIP. Despite countless attempts to carry out this experiment, our laboratory was only successful at generating samples with a sufficient amount of chromatin for ChIP a handful of times, making it difficult to statistically analyze our results and draw any significant conclusions. While the results we obtained from this research are interesting, there are likely better, more efficient means of chemically-induced nuclear reprogramming. That being said, one way to improve overall survival of cells following induction to reprogram towards pluripotency may be the use of antioxidants in culture systems. Several groups have reported success following inhibitory ROS exposure using antioxidants such as Vitamin C (Huangfu et al., 2008; Zhong-Dong Sui et al., 2016). Furthermore, the degree of variability in the effectiveness of treatment and the resulting outcome is, in our opinion, another great hindrance to the widespread application of this combination of small molecule inhibitors for priming BFF cells for nuclear reprogramming. We believed that the high variability in Oct-4, Nanog, and Sox-2 relative gene expression, as well as H3K4me3, H3K9me3, H3K27me3 we observed in BFF cells makes their response to pre-treatment with CHIR99021, PD325901, and NuP0178 unpredictable in addition to being inefficient.
LITERATURE CITED


Takatsu, K., M. Kuse, S. Yoshioka, and T. J. Acosta. 2015. Expression of epidermal growth factor (EGF) and its receptor in bovine endometrium throughout the luteal phase: effects of EGF on prostaglandin production in endometrial cells.


APPENDIX A: PROTOCOLS

DYNABEADS® RNA ISOLATION PROTOCOL

1. Harvest cells from culture flask as per standard procedure

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

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

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

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

6. Incubate at RT for 10 min in hybridization mixer

7. Place tube in magnetic separator for 2 min

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

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

10. Use sample directly for reverse transcription
cDNA SYNTHESIS PROTOCOL

1. Mix 4 µl of iScript reaction mix, 1 µl of reverse transcriptase, and 15 µl of sample mRNA in PCR tube (Bio-Rad Laboratories, Inc., Hercules, CA, USA)

2. Place tubes in the thermocycler and run for 5 min at 25°C, 30 min at 42°C, denaturation at 85°C for 5 min, and a final hold at 4°C.
qRT-PCR PROTOCOL FOR GENE EXPRESSION ANALYSIS

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

2. Pipette 18 µl of the appropriate master mix into the designated wells of a 96 multiwell plate tailor-made for Bio-Rad CFX Connect™ Real-Time PCR Detection System.

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

4. Cover plate with sealing foil.

5. Place plate in the Bio-Rad CFX Connect™ 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.
qRT-PCR PROTOCOL FOR ANALYSIS OF CHIP

1. Set up PCR reactions for the following templates: DNA from ChIP samples with test antibodies (H3K4me3, H3K9me3, H3K27me3), Input DNA samples, and DNA from ChIP performed with the negative control antibody (No Ab). Run PCR in duplicates.

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

3. For each PCR reaction, add 5 µl of sample DNA to 45 µl of the appropriate gene master mix.

4. Pipette 20 µl of the sample DNA + gene mix into the designated wells of a 96 multiwell plate tailor-made for Bio-Rad CFX Connect™ Real-Time PCR Detection System. Repeat this so that PCR is run in duplicates.

5. Cover plate with sealing foil

6. Place plate in the Bio-Rad CFX Connect™ instrument and run a denaturing cycle of 2 min at 94°C; 40 cycles of PCR (94°C for 30 sec and 52°C for 40 sec, 72°C for 30 sec); followed by final extension 72°C for 7 min.
CHROMATIN IMMUNOPRECIPITATION PROTOCOL OPTIMIZED FOR 3i-TREATED BFF CELLS USING ChIP-IT® EXPRESS MAGNETIC CHROMATIN IMMUNOPRECIPITATION KIT (Active Motif 53008)

Cell Fixation and Shearing

1. When cells are ready to harvest, freshly prepare the appropriate volume of Fixation Solution and 1X PBS Solution based on number of cells
2. Harvest cells by trypsinization per standard protocol. If cells were grown in multiple T-25 flasks, samples can be pooled at this time
3. Centrifuge 300 x g for 5 min
4. Discard supernatant and resuspend pellet in 5 mL of Fixation Solution
5. Incubate for 10 min at RT
6. Add 500 µL of 10X Glycine directly to cell suspension and mix to stop the fixation (100 µL 10X Glycine per 1 mL of Fixation Solution)
7. Centrifuge 300 x g for 5 min
8. Wash cells in 5 mL of ice-cold 1X PBS Solution
9. Discard supernatant and add 1 µL PIC and 1 µL PMSF to cell pellet. Store at -80°C

Shearing by Sonication

1. Thaw previously frozen cell pellet on ice and resuspend in 500 µL ice-cold Lysis Buffer supplemented with 2.5 µL PIC and 2.5 µL PMSF. Incubate on ice for 30 min
2. Transfer cell suspension to dounce homogenizer. Dounce on ice with 40-50 strokes to aid in nuclei release. Use a phase contrast microscope to monitor cell lysis after every 10 strokes to verify that the nuclei have been released
3. Transfer lysate to 1.7 mL microcentrifuge tube and centrifuge 2,400 x g for 10 min at 4°C
4. Carefully discard supernatant and resuspend in 300 µL Shearing Buffer supplemented with 1.5 µL PIC and 1.5 µL PMSF
5. Place tube in a rack holder and pack on ice, ensuring the tube is surrounded by ice
6. Shear DNA by sonication with a Cole Palmer Ultrasonic Processor (Cole-Palmer, Vernon Hills, IL USA) (500 watts, 120 volts, 3 mm probe) using the conditions determined to provide optimally sheared chromatin for BFF cells in this experiment (25 pulses of 20 sec, 30 sec rest).
7. Centrifuge 14,000 x g for 10 min at 4°C in a microcentrifuge
8. Very carefully transfer the supernatant, which contains the sheared chromatin, to a new 1.7 mL microcentrifuge tube
9. Prepare 50 µL aliquots of sheared chromatin. One 50 µL sample will be used immediately for assessing the efficiency of DNA shearing and determining the DNA concentration. Store the remaining 50 µL aliquots of sheared chromatin at -80°C

**DNA Cleanup to Assess Shearing Efficiency and DNA Concentration**
1. Add 150 µL dH2O and 10 µL 5 M NaCl to the sheared chromatin sample
2. Incubate tubes overnight at 65°C in thermocycler to reverse the cross-links
3. Following incubation, add 1 µL RNase A to each sample and incubate at 37°C for 15 min
4. Add 10 µL Proteinase K to each sample and incubate at 42°C for 1.5 h
5. Add 200 μL 1:1: Phenol/Chloroform TE saturated pH8 (Sigma Aldrich, P3803) to each tube and vortex to mix thoroughly
6. Centrifuge 14,000 x g for 5 min
7. Transfer the aqueous phase to a fresh microcentrifuge tube, then add 20 μL 3 M Sodium Acetate pH 5.2 followed by 500 μL 100% ethanol. Lastly, add 1 μL Glycogen (20 μg/μL) to aid in recovery of a visible pellet following precipitation
8. Incubate sample at -20°C overnight
9. The next day, centrifuge sample 14,000 x g for 10 min at 4°C in microcentrifuge
10. Carefully remove and discard the supernatant, leaving the pellet undisturbed
11. Add 500 μL 70% ice cold ethanol to the tube without disturbing the pellet and centrifuge 14,000 x g for 5 min at 4°C
12. Remove and discard supernatant, careful not to disturb the pellet. Allow pellet to air-dry under hood
13. Once dry, resuspend the pellet in 30 μL dH20
14. Determine the DNA concentration of the sheared sample using a spectrophotometer
15. To assess the efficiency of sonication, add 4 μL of a 6X Loading Buffer Dye to 16 μL of sheared chromatin. Load both 5 μL and 10 μL of sample on a 1% agarose gel and run at 100V for approximately 45 min. Optimal shearing by sonication yields a smear between 200-1500 bp.

**Immunoprecipitation**

1. If necessary, thaw 50 μL aliquot of chromatin on ice. Transfer 10 μL of chromatin to a microcentrifuge tube and set aside. This is the “Input DNA”, which will be
processed later and used as a control in PCR analysis. Store at 4° C if it will be used within 6 h; otherwise, store at -20° C.

2. Set up the ChIP reactions by adding the components shown in the table below to the provided siliconized 1.7 microcentrifuge tubes. Use the DNA concentration that was determined for the sheared chromatin sample to calculate the volume of chromatin to use. Resuspend the magnetic beads by inverting and/or vortexing the tube before use. The antibody should be the final component added to the reaction.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>One Reaction (if using less than 60 µL of chromatin)</th>
<th>One Reaction (if using more than 60 µL of chromatin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein G Magnetic Beads</td>
<td>25 µL</td>
<td>25 µL</td>
</tr>
<tr>
<td>ChIP Buffer 1</td>
<td>10 µL</td>
<td>20 µL</td>
</tr>
<tr>
<td>Sheared Chromatin (7-25 µg)</td>
<td>20-60 µL</td>
<td>61-100 µL</td>
</tr>
<tr>
<td>PIC</td>
<td>1 µL</td>
<td>2 µL</td>
</tr>
<tr>
<td>dH2O</td>
<td>Add enough so that final volume of reaction is 100 µL</td>
<td>Add enough so that final volume of reaction is 200 µL</td>
</tr>
<tr>
<td>Antibody</td>
<td>1-3 µg</td>
<td>1-3 µg</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>100 µL</strong></td>
<td><strong>200 µL</strong></td>
</tr>
</tbody>
</table>

3. Cap the tube and incubate overnight at 4° C on an end-to-end rotator.

4. Following incubation, spin the tube briefly to collect liquid from inside the cap.

5. Place tube on magnetic stand to pellet the beads on side of the tube.

6. Carefully remove and discard the supernatant.

**Wash Magnetic Beads**
1. Wash beads 1X with 800 µL of ChIP Buffer 1.

2. Wash beads 2X with 800 µL of ChIP Buffer 2. Allow no more than 1 min to elapse between removing buffer and adding the next wash.

3. After the final wash, remove as much supernatant as possible without disturbing the beads.

**Elute Chromatin, Reverse Cross-links, and Treat with Proteinase K**

1. Resuspend the washed beads in 50 µL Elution Buffer AM2, careful not to allow more than 1 min to elapse between removing ChIP Buffer 2 and adding the elution buffer.

2. Place the tube on an end-to-end rotator and incubate for 15 min at RT.

3. Briefly spin the tube to collect liquid from the cap.

4. Add 50 µL Reverse Cross-linking Buffer to the eluted chromatin and mix by pipetting up and down. Place tube in magnetic stand, and all the beads to pellet to the side of the tube.

5. Transfer the supernatant, which contains the chromatin, to a fresh tube.

6. Now, process the “Input DNA” by taking the 10 µL of chromatin that was set aside earlier and thaw on ice, if necessary. Add 88 µL ChIP Buffer 2 and 2 µL 5M NaCl to the Input DNA sample, so that the final volume is 100 µL.

7. Incubate both the Input DNA and ChIP samples at 95° C for 15 min in a thermocycler.

8. Remove the tubes from the thermocycler, allowing them to return to RT. Briefly spin the tubes if liquid has collected in the caps. Then, add 2 µL Proteinase K to each tube.
9. Mix well and incubate the tubes at 37° C for 1 h. During this time, remove the Proteinase K Stop Solution from the freezer, and allow it to sit at RT for 30 min to 1 h.

10. After incubation, allow the tubes to return to RT. Then, add 2 µL Proteinase K Stop Solution to each tube. Briefly centrifuge the tubes to collect any liquid from the caps. Immediately proceed with the DNA purification procedure, or store at -20° C until ready.
Purification of DNA from Chromatin IP Samples

1. If necessary, transfer ChIP samples to an Eppendorf tube, and add 5 volumes DNA Purification Binding Buffer for every one volume of sample DNA.

2. Add 5 µL 3M Sodium Acetate to each tube and mix. Check that the color of the DNA sample/DNA Purification Binding Buffer mixture is bright yellow, not light orange or violet. If it is light orange or violet, adjust the pH by adding more 3M Sodium Acetate- 5 µl at a time- until it is bright yellow in color.

3. For each sample, place a DNA purification column in the collection tube and add each sample to its own column. Close the cap on each column, place them with the collection tubes in a microcentrifuge, and spin 14,000 x g for 1 min at RT.

4. Remove the column from the collection tube, then remove and discard the flow-through from the collection tube.

5. Return the column to the collection tube, and add 750 µl 80% DNA Purification Wash Buffer (reconstituted with 100% ethanol) to the column.

6. Cap the column and centrifuge 14,000 x g for 1 min at RT.

7. Remove the column from the collection tube, then remove and discard the flow-through from the collection tube.

8. Return the column to the collection tube. With the column caps open, centrifuge again 14,000 x g for 2 min at RT to remove any residual DNA Purification Wash Buffer from the column. Discard the collection tube.

9. Transfer the column to a fresh Eppendorf tube, and add 50 µl DNA Purification Elution Buffer to the center of the column matrix. Wait for 1 min.
10. After 1 min, centrifuge the column with the collection tube 14,000 x g for 1 min at RT.

11. Discard the column. The DNA eluted into the Eppendorf tube is purified and ready to be used in PCR.
APPENDIX B: MEDIA FORMULATIONS AND STOCK SOLUTIONS

Complete Culture Medium

DMEM

10% FBS

1% P/S

3i+ Medium

DMEM

10% FBS

1% P/S

0.5 µM PD0325901

3 µM CHIR99021

1.8 µM NuP0178

Fixation Solution
VITA

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

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

Laura entered the School of Animal Sciences' graduate program in reproductive physiology at Louisiana State University under the direction of Dr. Kenneth R. Bondioli. She received a Master of Science degree in this program in 2010. Laura continued her education within this program and is now a candidate for the Doctor of Philosophy degree in reproductive physiology in the School of Animal Sciences at Louisiana State University under the guidance of Dr. Kenneth R. Bondioli. Laura is currently employed by Ovation Fertility™ and works as an Andrologist and Embryologist at Fertility Answers® in Baton Rouge, Louisiana.