REGULATION AND EXPRESSION OF NANOG, OCT4, AND SOX2 IN THE
BOVINE BLASTOCYST FOLLOWING SOMATIC CELL NUCLEAR TRANSFER

by

Justin Scott Hall

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Animal Science

Approved:

Kenneth L. White
Major Professor

Abby Benninghoff
Committee Member

Thomas D. Bunch
Committee Member

Kenneth L. White
ADVS Department Head

Mark R. McLellan
Vice President for Research
Dean of the School of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2013
ABSTRACT

Regulation and Expression of Nanog, Oct4, and Sox2 in the Bovine Blastocyst Following Somatic Cell Nuclear Transfer

by

Justin Scott Hall, Master of Science
Utah State University, 2013

Major Professor: Dr. Kenneth L. White
Department: Animal, Dairy, and Veterinary Sciences

A live birth from a somatic cell nuclear transfer (SCNT) embryo represents a small percentage of donor cells that survived the reprogramming gauntlet. The inability to reprogram histone modifications in the donor cell line could add to the reprogramming deficiencies associated with SCNT. The effects of two histone modifications associated with transcriptional activation (H3K4m3 and H4K16ac) and two histone modifications associated with repressing transcription (H3K9m2 and H3K27me3) were evaluated in the context of their association to three genes known to contribute to maintaining totipotency: Nanog, Oct4, and Sox2. A μChIP assay was utilized using antibodies specific for each histone modification followed by real time PCR (qPCR) analysis to quantify the percentage of each gene associated with each particular histone modification. Gene expression analysis was followed by immunofluorescence and protein analysis. Results of these analyses suggest that gene association to certain histone modifications did not accurately predict gene expression in bovine blastocyst embryos. Of the three genes
studied, only Oct4 expression differed significantly between in vitro fertilized (IVF; control) and SCNT blastocysts. Protein levels detected through immunofluorescence correlated directly with the gene expression analysis. Nanog and Sox2 expression profiles of IVF and SCNT bovine blastocysts are similar, yet the histone modification profiles associated with all three genes differ significantly. Altered expression levels in developmentally important genes will likely result in abnormal activity of the associated cellular pathway. Aberrant histone modifications, along with abnormal Oct4 expression, may contribute to the low percentage of SCNT embryos that result in live offspring.
Regulation and Expression of the Nanog, Oct4, and Sox2 Genes in the Bovine Embryo Following Somatic Cell Nuclear Transfer

Justin S. Hall Master of Science

The Animal, Dairy, and Veterinary Sciences Department (ADVS) and the Center for Integrated Biosystems (CIB) at Utah State University are studying various molecular mechanisms involved in the animal cloning process. This study involves the extensive network of people, facilities, equipment, and funding already associated with the CIB and ADVS joint project.

Cloning involves many molecular challenges that for the most part have become roadblocks for the normal development of the fetus. The mechanisms necessary to transform an adult cell into a competent stem cell that can then transform and develop into a healthy organism are poorly understood. Some of these roadblocks have been broadly defined. In this study histone modifications are examined in terms of how they might influence the expression of three developmentally important genes (Nanog, Oct4, and Sox2). Understanding the molecular mechanisms and their role in histone modifications and subsequent gene expression in early development will lead to identifying genetic deficiencies that contribute to the poor success in animal cloning.

Currently animal cloning is very inefficient, although the benefits associated with the science involved are limitless. Successful cloning has the potential to provide newer and better biopharmaceuticals, and animal models for human diseases; produce superior livestock; save endangered species; and contribute to stem cells research.
ACKNOWLEDGMENTS

I would like to thank Dr. Kenneth L. White for giving me the opportunity to complete this project in his laboratory and under his supervision. I would also like to thank my committee members, Drs. Thomas Bunch and Abby Benninghoff, for their support, critiques, and assistance throughout this entire process.

Along with my committee I would like to thank those directly involved or collaborating with our lab on this project, Drs. Chris Davies, Clay Isom, Heloisa Rutigliano, Aaron Thomas, and Qinggang Meng, and the ever helpful Ben Sessions, Ammon Bayles, Kira Perry, and Aaron Davis, who all made laboratory work enjoyable.

Lastly I would like to thank my family. My wife, Sherrie, and two boys, Koy and Titan, are my everything. Without them I would not have ever finished.

This work was supported by USDA grant # 090867.

Justin S. Hall
## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABSTRACT</strong></td>
<td>iii</td>
</tr>
<tr>
<td><strong>PUBLIC ABSTRACT</strong></td>
<td>v</td>
</tr>
<tr>
<td><strong>ACKNOWLEDGMENTS</strong></td>
<td>vi</td>
</tr>
<tr>
<td><strong>LIST OF TABLES</strong></td>
<td>ix</td>
</tr>
<tr>
<td><strong>LIST OF FIGURES</strong></td>
<td>x</td>
</tr>
<tr>
<td><strong>LIST OF ABBREVIATIONS</strong></td>
<td>xi</td>
</tr>
<tr>
<td><strong>LITERATURE REVIEW</strong></td>
<td>1</td>
</tr>
<tr>
<td>Epigenetics</td>
<td>1</td>
</tr>
<tr>
<td>History of Cloning</td>
<td>6</td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>10</td>
</tr>
<tr>
<td><strong>RESULTS</strong></td>
<td>13</td>
</tr>
<tr>
<td>μChIP</td>
<td>13</td>
</tr>
<tr>
<td>Gene Expression</td>
<td>17</td>
</tr>
<tr>
<td>Protein Analysis</td>
<td>18</td>
</tr>
<tr>
<td><strong>DISCUSSION</strong></td>
<td>24</td>
</tr>
<tr>
<td><strong>MATERIALS AND METHODS</strong></td>
<td>28</td>
</tr>
<tr>
<td>Oocyte Collection and Maturation</td>
<td>28</td>
</tr>
<tr>
<td>IVF Embryo Production</td>
<td>28</td>
</tr>
<tr>
<td>SCNT Embryo Production</td>
<td>29</td>
</tr>
<tr>
<td>μChIP Chromatin Preparation</td>
<td>29</td>
</tr>
<tr>
<td>μChIP Antibody-bead Complexes</td>
<td>31</td>
</tr>
<tr>
<td>Purification of Input and Precipitated Chromatin</td>
<td>32</td>
</tr>
<tr>
<td>Quantitative PCR</td>
<td>33</td>
</tr>
<tr>
<td>RNA Extraction and Reverse Transcription</td>
<td>34</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>34</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>35</td>
</tr>
<tr>
<td><strong>REFERENCES</strong></td>
<td>38</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Primers used for µChIP PCR……………………………………..33</td>
</tr>
<tr>
<td>2</td>
<td>Primers used for gene expression analysis………………..34</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Association of DNA with Histone 3 Lysine 4 tri-methyl (H3K4me3)</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>Association of DNA with Histone 4 Lysine 16 acetylation (H4K16ac)</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>Association of DNA with Histone 3 Lysine 9 di-methyl (H3K9me2)</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>Association of DNA with Histone 3 Lysine 27 tri-methyl (H3K27me3)</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>Delta-Delta Ct values for RT-PCR</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>Nanog staining in IVF and SCNT blastocysts</td>
<td>19</td>
</tr>
<tr>
<td>7</td>
<td>Oct4 staining in IVF and SCNT blastocysts</td>
<td>20</td>
</tr>
<tr>
<td>8</td>
<td>Sox2 staining in IVF and SCNT blastocysts</td>
<td>21</td>
</tr>
<tr>
<td>9</td>
<td>Sox2 and Oct4 in IVF and SCNT blastocysts</td>
<td>22</td>
</tr>
<tr>
<td>10</td>
<td>Negative Control with non relevant Ig</td>
<td>22</td>
</tr>
<tr>
<td>11</td>
<td>Immunofluorescence and protein analysis</td>
<td>23</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔΔCt</td>
<td>delta-delta Ct</td>
</tr>
<tr>
<td>ΔCt</td>
<td>delta Ct</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>trimethylated histone H3-lysine 4</td>
</tr>
<tr>
<td>H3K27me2</td>
<td>dimethylated histone H3-lysine 27</td>
</tr>
<tr>
<td>H3K9me2</td>
<td>dimethylated histone H3-lysine 9</td>
</tr>
<tr>
<td>H4K16ac</td>
<td>acetylated histone H4-lysine 16</td>
</tr>
<tr>
<td>HATs</td>
<td>histone acetyl transferases</td>
</tr>
<tr>
<td>HDACs</td>
<td>histone deacetylases</td>
</tr>
<tr>
<td>HMTs</td>
<td>histone methyltransferases</td>
</tr>
<tr>
<td>ICM</td>
<td>inner cellular mass</td>
</tr>
<tr>
<td>iPS</td>
<td>induced pluripotent</td>
</tr>
<tr>
<td>IVF</td>
<td>in vitro fertilization</td>
</tr>
<tr>
<td>NT</td>
<td>nuclear transfer</td>
</tr>
<tr>
<td>SCNT</td>
<td>somatic cell nuclear transfer</td>
</tr>
<tr>
<td>TFs</td>
<td>transcription factors</td>
</tr>
<tr>
<td>μChIP</td>
<td>micro chromatin immuno-precipitation</td>
</tr>
</tbody>
</table>
LITERATURE REVIEW

Epigenetics

Epigenetics is the study of heritable changes in gene expression due to components other than the alteration of the genetic sequence, such as changes to DNA methylation marks and modifications to the histone tails (Jaenisch and Bird, 2003). For example, the human body has over 200 cell types, with each cell possessing the same unique DNA sequence but differing in gene expression. These epigenetic influences aid in creating different cell and tissue types by affecting the expression patterns of specific genes. Interestingly, genetically identical twins develop different epigenetic markers over time. Thus, different phenotypes can stem from identical genotypes (Fraga et al., 2005). Some scientists indicate that epigenetic modifications have evolved as a genome defense against parasitic and viral sequences (Matzke et al., 1999). During early embryonic development, cellular differentiation occurs as a consequence of epigenetic influences on the genome. These epigenetic modifications regulate the expression of cell-type specific genes. If epigenetics governs the genes associated with each cell and tissue type, then somatic cell nuclear transfer (SCNT) is successful only when the donor cell genome can be reprogrammed sufficiently to give rise to every cell-type in the resultant organism, including extra-embryonic membranes. Incomplete or abnormal epigenetic reprogramming of the donor cell genome has been shown to contribute to the low efficiency of SCNT and is believed to contribute to the phenotypic abnormalities associated with the resultant offspring.

One important epigenetic mechanism of gene regulation deals with the chemical modification of the histone tails. Histone proteins are an integral part of DNA packaging.
and chromosome structure. A basic unit of packaged DNA is called a nucleosome, which consists of 146 base pairs of DNA wrapped around an octamer of four core histone proteins (H3, H4, H2A, and H2B) (Luger et al., 1997). The N-terminus tail of the histone protrudes outwardly from the nucleosome and contains multiple modification sites that are available for interactions with other proteins. At the N terminus of each histone are 15-38 amino acids that form the “tails,” which provide multiple sites for post-translational modifications to the underlying DNA sequence. These modifications include acetylation, methylation, ubiquitination, phosphorylation, and sumoylation (Goll and Bestor, 2002). Histone modifications are accomplished through the function of nuclear enzymes such as histone acetyltransferases (HATs), deacetylases (HDACs), and methyltransferases (HMTs). There are a great many potential combinations of modifications to the 38 amino acids forming the histone tails. The characterization of the biological events associated with each unique combination is daunting, yet some patterns have emerged. Knowledge of specific histone profiles will invariably enhance our understanding of the reprogramming necessities associated with successful SCNT.

Specific histone modifications have been shown to be reversible and related to gene expression and regulation. Methylation of particular residues of the histone tail is a key modification for regulating chromatin packaging and gene expression (Goll and Bestor, 2002; Barski et al., 2007). Trimethylated histone H3-lysine 4 (H3K4me3) is associated with gene activation, whereas dimethylated histone H3-lysine 9 (H3K9me2) and trimethylated histone 3 H3-lysine 27 (H3K27me3) are predominantly linked with transcriptional repression. Acetylation of histone H4 on ysine 16 (H4K16Ac) aids in the regulation of chromatin folding and transcriptional activation (Shogren-Knaak et al.,
2006; Wang et al., 2008; Rosenfeld et al., 2009). While most histone modifications have been grouped according to their function to either silence or activate transcription, conflicting modifications have also been shown to co-exist. The most notable example occurs in the regulatory loci of developmentally important genes in embryonic stem cells (ES), where competing modifications co-localize, producing a bivalent domain of both activating and repressing modifications (Bernstein et al., 2006; Rugg-Gunn et al., 2010).

Some researchers have hypothesized that this epigenetic pattern is present in genes where a rapid transition between activation and repression would be advantageous, mainly in association with embryonic development and determining cell fate (Ku et al., 2008; Sevostyanova et al., 2008).

In connection with cell lineage determination, countless efforts have been made to establish the epigenome state that maintains pluripotency. The expression pattern of pluripotent cells is unique, and pinpointing the epigenetic mechanisms involved in establishing pluripotency will lead to many new biomedical applications, from organ regeneration to more efficient SCNT. ES cells are the progenitors to hundreds of cell types, and their plasticity is at the heart of the developmental research of today. In 1923 a Russian scientist named Maximow was studying the effects of x-rays on inflammation and noted how some cells were able to differentiate into other cells and were “endowed with great prospective potencies of development” (Maximow, 1923). In 1960 the same idea of radiation on mice bone marrow cells fueled experiments that led to the term “stem cells” (McCulloch and Till, 1960). In 1981 cells from the inner-cellular mass (ICM) of an early mouse embryo were isolated and termed “embryonic stem cells,” and by 1998 a group at the University of Wisconsin-Madison was successful in isolating stem cells from
human embryos (Martin, 1981; Thomson et al., 1998). In 2007 the Nobel Prize was awarded for the knockout mouse project, which gave researchers the ability to study gene function in live mice (Austin et al., 2004). Despite some public discomfort on the topic of human embryonic stem cells for research, and the multitude of complexities in genomics, adult cells can now be reprogrammed into putative stem cells and used in select therapeutics to rejuvenate damaged or diseased tissue types.

The mechanisms and components of establishing a pluripotent state are complex, yet they are becoming more defined daily. A hallmark of pluripotency is the presence of pluripotent transcription factors (TFs) (Yamanaka and Takahashi, 2006). Namely, Oct4, Nanog, and Sox2 are TFs that are hallmarks of establishing a pluripotent state (Yamanaka and Takahashi, 2006; Pan and Thomson, 2007).

The gene Nanog was named by the Scottish professor Ian Chambers as a reference to the legendary “Tir Na Nog” land of perpetual youth. In 2003 they classified Nanog in the “transcription factor hierarchy that defines ES cell identity” (Chambers et al., 2003). Nanog is involved in the self-renewal of pluripotent ES cells and its absence in mouse ES cells promotes differentiation (Yates and Chambers, 2005). In primates, siRNA was used to knockdown Nanog, which resulted in differentiation of ES cells (Yasuda et al., 2006). Nanog has also been shown to work in concert with other TFs (Chambers, 2004), although Takahashi and Yamanaka initially produced induced Pluripotent Cells (iPS) without citing Nanog as a necessary TF. Nevertheless, Nanog remains at the core of epigenetic research today.

Oct4 is one TF that binds to the “oct”amer sequence ATTTGCAT and is synonymous with POU5F1 (Petryniak et al., 1990). The function of Oct4 is well
documented. Nichols and coworkers showed that Oct4 mRNA is found in all early cleavage stage mouse blastomeres (Nichols et al., 1998). This same group went on to report that Oct4 expression transitions specifically to the ICM cells during pre-implantation embryogenesis. In 2005 RNA interference was used to knockdown Oct4 and Nanog, leading to cell differentiation in human ES cells (Zaehres et al., 2005). Oct4-deficient embryos will develop to blastocyst, however the ICM cells are not pluripotent (Nichols et al., 1998). In 2004 Oct4 knockout mice were produced (Kehler et al., 2004). This group showed that Oct4 is expressed in the primordial germ cells of mice, and knockout of this gene caused apoptosis to the primordial germ cells, resulting in sterility. Oct4 was also one of four necessary TFs shown to induce pluripotency by Yamanaka (Yamanaka and Takahashi, 2006).

Sox2 is another key TF cited by Yamanaka to contribute to pluripotency (Yamanaka and Takahashi, 2006). As early as 1995, Sox2 was linked with Oct4. The two TFs were observed to form a joint complex that promotes the transcription of Fibroblast Growth Factor 4, which is expressed in the ICM of the mouse blastocyst (Yuan et al., 1995). In 2007 Masui et al demonstrated that inducible Sox2-null ES cells were still able to activate the genes previously classified as “Oct-Sox” enhanced. This same group identified the regulatory role of Sox2 in Oct3/4 expression, thus demonstrating the main function of Sox2 in maintaining appropriate Oct3/4 expression (Masui et al., 2007). Sox2 and Oct4 also interact with the Nanog promoter, linking these three as essential TFs in the mechanisms associated with maintaining pluripotency (Rodda et al., 2005). Additional details of these three TFs emerge daily due to their unparalleled role in maintaining a pluripotent state. While other genes and factors have also been shown to
contribute to the pluripotent state, these three TFs are sufficient representatives of this developmentally important state for the present study.

**History of Cloning**

The nuclear transfer (NT) process was first proposed by Hans Spemann, the German Nobel Laureate, in 1938, although he lacked the necessary equipment to sufficiently test the hypothesis (Spemann, 1938). Cloning by NT involves removing the DNA (enucleation) from an unfertilized oocyte, followed by the addition of foreign DNA to the enucleated oocyte (cytoplast), yielding a zygote with a full compliment of DNA that is capable of producing a living organism. Although the concept of manually removing the oocyte DNA and replacing it with another cell’s DNA appears relatively simple, the molecular mechanisms involved in remodeling and reprogramming the donor cell genome after entry into the cytoplasm of the cytoplast remain somewhat complex and undefined.

In 1952 Briggs and King successfully produced a Northern Leopard Frog using the NT process (Briggs and King, 1952). This same group later concluded that NT embryos produced using cells from more developed embryos had increasingly poor developmental potential (King and Briggs, 1956). Multiple somatic cell types, or non-sex cells from the body of an organism, were used in many of the first SCNT attempts in frogs. These somatic cell sources included lymphocytes (Wabl et al., 1975), skin (Gurdon et al., 1975), erythrocytes (Di Berardino and Hoffner, 1983), and erythroblasts and leukocytes erythroblasts (Di Berardino and Orr, 1992). These experiments yielded tadpoles that were normal morphologically, proving that some somatic cell types are capable of de-differentiating. Although successful, no adults were produced.
By the 1980’s, scientists studying mammalian developmental biology began using the NT method to generate embryos. Some of the first experiments involved mice, where pronuclei were transferred to enucleated zygotes (McGrath and Solter, 1984). This same experiment resulted in live births from embryos produced using pronuclei, but failed to produce live births using nuclei from the two-cell, four-cell, eight-cell, or ICM. Live NT lambs were produced using eight and sixteen-cell blastomeres as nuclear donors in 1986 (Willadsen, 1986). This work was followed in 1987 by the birth of the first NT cattle (Robl et al., 1987). Mice, rats, goats, monkeys, rabbits, and pigs were also produced using cells from preimplantation embryos (Di Berardino, 2001). Although using these embryonic cells proved successful, NT imploring stem cell-like donor cells was not (Tsunoda and Kato, 1993; Stice et al., 1996). In 1994 Sims and First produced cloned calves by transferring inner cell mass (ICM) cells that had been cultured in vitro for up to 28 days (Sims and First, 1994). In summary, NT mammalian clones were produced using pronuclei, giving rise to the use of cells from preimplantation embryos, which was then followed by the use of cultured ICM (differentiated) cells, and all of this work ultimately led to the use of an adult cell (mammary) and the birth of Dolly (Wilmut et al., 1997). Using donor cells from mammary tissue was not particularly advantageous, however they were differentiated-adult cells. Dolly’s birth has been followed by the live birth of many mammalian species using NT of somatic cells including ferrets (Li et al., 2006b), dogs (Lee et al., 2005), horses (Galli et al., 2003), cats (Shin et al., 2002), rats (Zhou et al., 2003), rabbits (Chesne et al., 2002), mules (Woods et al., 2003), pigs (Polejaeva et al., 2000), goats (Baguisi et al., 1999), cattle (Wells et al., 1999), and mice (Kishigami et al., 2006). Results of these experiments show that the unfertilized oocyte contains the
components necessary to take a terminally differentiated somatic cell and reverse the differentiating process sufficiently to produce the many different cell types of a complex organism. Although the use of adult somatic cells has been successful, attempts to use stem cell-like lines in the NT process are less effective (Sung et al., 2006). This reduced efficiency using adult stem cells is puzzling, and more research is needed to fully characterize the molecular factors associated with the donor cell’s epigenetic state that will give rise to the most successful SCNT outcome.

The proficiency of the SCNT process coupled with the percentage of SCNT pregnancies that result in the birth of live offspring reveals that there is room to refine the overall process. Aside from the many different protocols in embryo transfer, cell culture, and data representation, to say nothing of the differences that may arise from the *bos taurus* gene pool potpourri, less than 5% of transferred cloned embryos result in live births (Oback and Wells, 2003). Determining the specific deficiencies present in the other 95% of transferred failed SCNT embryos is daunting, yet specific abnormalities have been identified. One such deficiency is abnormal placentation and enlarged placentomes (Constant et al., 2006). Others cite epigenetic abnormalities in the cloned embryo, including improper DNA methylation and aberrant histone modifications as possible hurdles for proper embryonic development (Arney et al., 2001; Reik et al., 2001; Santos and Dean, 2004; Steele et al., 2005; Eilertsen et al., 2007). The multiplicity of mechanisms occurring during normal embryogenesis and development is overwhelmingly complex, yet the generation of live offspring from SCNT proves that sufficient successful epigenetic reprogramming and subsequent development is possible.
The research presented in this Master’s Thesis combines three pluripotent TFs
\( (\text{Nanog}, \text{Oct4}, \text{and Sox2}) \) with four well-defined histone modifications (H3K4me3, H3K9me2, H3K27me3, and H4K16Ac) and examines their regulatory role associated with gene expression following SCNT in the bovine blastocyst. The main objective in this undertaking was to determine any abnormalities with the four histone modifications listed above and their association to \( \text{Nanog}, \text{Oct4}, \) and \( \text{Sox2} \), and how these abnormalities affect gene expression and ultimately protein levels. We hypothesized that the histone modification profiles in SCNT embryos associated with these genes will more closely resemble that of the donor cell. Further we postulated that the failure to completely reprogram the donor cell will lead to altered gene expression and protein levels. The new knowledge obtained from these studies will help identify the epigenetic differences that might be associated with these three genes in a cloned blastocyst and demonstrate how those abnormalities affect the expression of these developmentally important genes.
INTRODUCTION

Since the birth of Dolly in 1997, SCNT has been successful in producing live offspring in a number of different species. The benefits of the nuclear transfer technology extend far beyond producing animals, and new applications for the science continue to emerge. Live births from SCNT provide excellent evidence that the oocyte contains the components necessary to transform an adult cell into a stem cell state and ultimately differentiate into all of the cell types required to produce viable offspring. The molecular mechanisms involved in this transformation are currently undefined, and tremendous effort is being expended to better characterize this phenomenon. Although SCNT is successful, the percentage of SCNT embryos that result in live and healthy offspring is quite low. Only 5-10% of transferred bovine embryos result in live births (Oback and Wells, 2007). These percentages leave substantial opportunity for improvement, and a correct understanding of the mechanisms associated with this cellular transformation should not only improve efficiency and live birth rates, but will further enhance the broader applications of this technology.

One of the proposed inefficiencies of the SCNT process is associated with what has been referred to as nuclear reprogramming. Although the oocyte has the capacity to program a fertilizing sperm nucleus during traditional fertilization, incomplete reprogramming of the differentiated donor-cell nucleus has been cited as a key factor for the low success rate of SCNT (Bourc'his et al., 2001; Beyhan et al., 2007). Specifically, the differences between SCNT and traditionally fertilized embryos in DNA methylation and histone modifications are documented abnormalities (Bourc'his et al., 2001; Kang et al., 2002; Enright et al., 2003). These epigenetic irregularities are typically accompanied
by a concomitant aberrant gene expression profile in SCNT embryos (Bortvin et al., 2003; Smith et al., 2005; Li et al., 2006a; Beyhan et al., 2007). Other irregularities in SCNT between pregnancy establishment and birth include hydroallantois (Lawrence et al., 2005), respiratory distress, stillbirth (Hill et al., 1999), and abnormal placentation (Constant et al., 2006). Abnormalities in gene expression and overall development are certainly manifestations of earlier anomalies, and pinpointing normal and necessary epigenetic components is a necessary step in correcting these irregularities.

While some scientists attempt to trace the mechanisms associated with normal gene expression and development, others have successfully pursued the reverse approach, whereby an adult cell is reprogrammed into an embryonic-like stem cell without exposure to the cytoplasmic environment of the unfertilized oocyte. The mechanisms of establishing a stem cell state are complex, but some hallmarks do exist. Yamanaka and Takahashi found that introducing four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) to fibroblast cultures resulted in pluripotent stem cells (Yamanaka and Takahashi, 2006). Another important factor, Nanog, was later shown to act in concert with Oct4 and Sox2 as down-stream regulators on other genes apparently important to establishing a ES cell state (Pan and Thomson, 2007). These TFs work together to properly regulate their own expression levels, ultimately maintaining stem cell-type characteristics.

In the present study, we sought to better understand the inefficient process of SCNT, the epigenetic irregularities in SCNT embryos, the importance of Nanog, Oct4, Oct4, and Sox2 in establishing and maintaining ES cells, and the potential associated with refining the SCNT process. Specifically, we set out to characterize the effect of four histone modifications on the gene expression of three pluripotent TFs in the bovine
blastocyst and define any irregularities that are present in SCNT embryos. A correct characterization of the epigenetic mechanisms associated with reprogramming adult cells into embryonic cells will further propel the application of the stem cell biology.
RESULTS

µChIP

The three genes in question (Nanog, Oct4, and Sox2) and their association with four histone modifications (H3K4me3, H3K9me2, H3K27me3, and H4K16ac) and with four DNA sources (donor cells, IVF, SCNT, and trophoblast cells) were analyzed using a two-way ANOVA comparison. Results within each gene and histone modification were compared across all cell types, and many significant differences were apparent. Broadly, these histone modifications are only minimally associated with the three genes in question in trophoblast cells, with five data points less than 1%, and the other seven trophoblast data points below 16% of the total. We were not able to detect association of Oct4 with any of the histone modifications in question in any cell type, with the highest percentage of the total being 9% in trophoblast cells.

Two-way ANOVA followed by Bonferroni’s post-hoc means test further classified the interaction and variance in the samples. This test accounted for the variance due to the DNA source, the gene, and the interaction between the DNA source and the gene. Analysis of each histone modification showed that the DNA source accounted for the highest percentage of the variance seen, specifically 42% in H3K4me3, 31% in H3K9me2, 36% in H3K27me3, and 38% in H4K16ac. The effect of the gene and the interaction between the gene and DNA source both accounted for less than 25% of the variance seen with each modification.

H3K4me3 showed significant differences between donor cells, IVF, and SCNT in Nanog, where 46% of SCNT DNA and only 10% of IVF DNA was precipitated. Differences in Sox2 were seen in the same cell types, where again half of SCNT DNA
and 25% of IVF DNA were pulled down. We obtained a similar pattern with H4K16ac that we observed with the related H3K4me3 modification, and both are associated with transcriptional activation. In evaluating Nanog, 60% of SCNT and 20% of IVF DNA was precipitated, and 30% of both DNA sources were precipitated in Sox2.

The two histone modifications associated with repressing transcription, H3K9me2 and H3K27me3, exhibited similar patterns in both Nanog and Sox2 in regards to IVF, donor cells, and SCNT samples. In fact, both repressive and activating modifications exhibit somewhat similar patterns of association with all three genes evaluated, rather than observing an inverted pattern. This pattern is consistent with other published reports, where bivalent domains are present in developmentally important genes (Rugg-Gunn et al., 2010). Our initial hypothesis was that the donor cells used in SCNT experience incomplete reprogramming, and that the histone profiles of the resultant cells from SCNT embryos would more closely resemble the profiles of the donor cell. These results clearly indicate that the reprogramming associated with these four histone modifications and three genes is incomplete in relation to some genes and modifications, and adequate in relation to others. The histone profiles of SCNT embryos resemble those of the donor cell in some instances, yet in other locations are similar to the IVF control embryos.
**FIGURE 1.** Association of DNA with Histone 3 Lysine 4 tri-methyl (H3K4me3). Values represent the average of four independent \( \mu \)ChIP assays using 10 day-7 blastocysts or 1,000 cells, normalized by the amount of input DNA. Different superscripts within individual genes represent significant differences \((P<0.05)\) as determined by two-way ANOVA with Bonferroni post-hoc test comparisons. This histone modification is associated with transcriptional activation.

**FIGURE 2.** Association of DNA with Histone 4 Lysine 16 acetylation (H4K16ac). Values represent the average of four independent \( \mu \)ChIP assays using 10 day-7 blastocysts or 1,000 cells, normalized by the amount of input DNA. Different superscripts within individual genes represent significant differences \((P<0.05)\) as determined by two-way ANOVA with Bonferroni post-hoc test comparisons. This histone modification is associated with transcriptional activation.
FIGURE 3. Association of DNA with Histone 3 Lysine 9 di-methyl (H3K9me2). Values represent the average of four independent μChIP assays using 10 day-7 blastocysts or 1,000 cells, normalized by the amount of input DNA. Different superscripts within individual genes represent significant differences ($P<0.05$) as determined by two-way ANOVA with Bonferroni post-hoc test comparisons. This histone modification is associated with transcriptional repression.

FIGURE 4. Association of DNA with Histone 3 Lysine 27 tri-methyl (H3K27me3). Values represent the average of four independent μChIP assays using 10 day-7 blastocysts or 1,000 cells, normalized by the amount of input DNA. Different superscripts within individual genes represent significant differences ($P<0.05$) as determined by two-way ANOVA with Bonferroni post-hoc test comparisons. This histone modification is associated with transcriptional silencing.
Gene Expression

The expression of Nanog, Oct4, and Sox2 were analyzed in IVF and SCNT blastocysts (Figure 5). Three pools of each embryo type were analyzed. After computing $\Delta\Delta$Ct values and identifying the fold change in expression, significantly ($P<0.05$) higher levels of Oct4 were present in IVF than SCNT blastocysts. Expression of Nanog and Sox2 in SCNT blastocysts did not differ significantly compared to IVF controls ($P=0.2916$, $P=0.2417$ respectively).

FIGURE 5. Delta-Delta Ct values for RT-PCR. Values represent the average of three replicates, where RNA was extracted from a pool of 10 day-7 blastocysts. Asterisk indicates significant difference ($P=0.0402$), as determined by the Student’s $t$-test.
**Protein Analysis**

Embryos were imaged using a 20x plan-APO objective. The AxioVision software was used to quantify the fluorescence seen in the stained embryos. This calculation included the total number of pixels in the image that were stained positive for DAPI, divided by the total number of pixels positive for each fluorescent tag. Thus the amount of nuclei in each embryo that contain the protein of interest is expressed as a percentage of the total number of nuclei. The student’s $t$-test was applied to these percentages, and the average percentages of ten embryos for each protein and embryo type is represented in Table 6. On average, IVF blastocysts contained a higher percentage of positive nuclei. Of the TFs in question, only Oct4 was considered significantly different in SCNT blastocysts, with a $P$-value of 0.0119. Nanog and Sox2 values were similar, with $P$-values of 0.4416 and 0.5847. This quantitative data (Figure 11) is supported visually by the images produced below (Figures 6-10). This protein expression pattern also coincides with the mRNA results discussed previously.
Figure 6. Nanog staining in IVF and SCNT blastocysts. Images are representative of Nanog in IVF (A-C) and SCNT (D-F) blastocysts. Ten embryos were imaged for each type (IVF or SCNT) and protein (Nanog, Oct4, or Sox2). These images are indicative of the average percentage of nuclei positive for Nanog in IVF (44%) and SCNT (40%), calculated by dividing the total # of pixels positive for DAPI (A, D) by the total # of pixels positive for the antibody/fluorophore (B, D). The light blue color created by the merged images (C, F) indicates the presence of Nanog in the nuclei of the embryonic cells, and most positive cells are in the ICM. Results of the statistical analyses of immunofluorescence data are provided in Figure 11.
FIGURE 7. Oct4 staining in IVF and SCNT blastocysts. Images are representative of Oct4 in IVF (A-C) and SCNT (D-F) blastocysts. Ten embryos were imaged for each type (IVF or SCNT) and protein (Nanog, Oct4, or Sox2). These images represent the average percentage of nuclei positive for Oct4 in IVF (46%) and SCNT (32%) calculated by dividing the total # of pixels positive for DAPI (A, D) by the total # of pixels positive for the antibody/fluorophore (B, D). The light blue present in the merged images (C, F) indicates the presence of Oct4 in the nuclei of the embryonic cells, and most positive cells are in the ICM. Results of the statistical analyses of immunofluorescence data are provided in Figure 11.
FIGURE 8. Sox2 staining in IVF and SCNT blastocysts. Images are representative of Sox2 in IVF (A-C) and SCNT (D-F) blastocysts. Ten embryos were imaged for each type (IVF or SCNT) and protein (Nanog, Oct4, or Sox2). These images represent the average percentage of nuclei positive for Sox2 in IVF (43%) and SCNT (40%) calculated by dividing the total # of pixels positive for DAPI (A, D) by the total # of pixels positive for the antibody/fluorophore (B, D). The pink color in the merged images (C, F) indicates the presence of Sox2 in the nuclei of the embryonic cells, and most positive cells are in the ICM. Results of the statistical analyses of immunofluorescence data are provided in Figure 11.
FIGURE 9. Sox2 and Oct4 in IVF and SCNT blastocysts. Images show co-localization in IVF (A-D) and SCNT (E-H) blastocysts of Sox2 (B, F) and Oct4 (C, G).

FIGURE 10. Negative control performed with non relevant Ig. Images show Hoechst (A), non relevant IgG and Alexa Fluor 488 (B) and merged (C).
Figure 11. Immunofluorescence and protein analysis. Data was normalized by dividing the total number of positive pixels for DAPI by the total number of positive pixels containing each antibody/fluorophore in each image. Values represent the average of ten day-7 blastocysts and an asterisk represents significant difference (P=0.012), as determined by the Student’s t-test.
DISCUSSION

This study provides a comparative analysis of prominent histone modifications and their effect on the expression of three key pluripotent genes. The ability to follow the histone profiles of the donor cell from fibroblast to SCNT blastocyst is unique to this model. This study represents the first attempt to couple the association of these histone modifications in these three genes with a quantification of the relative protein expression in the bovine model. The histone patterns of SCNT embryos mimic both those seen in the donor cells and IVF embryos, indicating a reprogrammed hybrid of the progenitor fibroblast and the typical developing blastocyst. Thus, the hypothesis that SCNT blastocysts would retain histone modification profiles resembling those of donor cells instead of control IVF blastocysts was correct for some loci, whereas other loci acquired the modifications observed in appropriately programmed IVF embryos.

Significant differences in the percentage of DNA associated with each of the four histone modifications examined were observed when comparing DNA obtained from the donor cell to that from SCNT embryos. IVF and SCNT profiles were similar for the Sox2 gene and its association with three of the four histone modifications we examined. Similarly in Nanog, three of the four histone modification profiles of IVF embryos resembled those of the donor cells. Other observations from our group illustrate the role of DNA methylation in regulating the expression of these three genes (Davis et al., unpub. obs). Specifically, the methylation patterns in SCNT blastocysts observed in Nanog and Sox2 were more similar to the methylation patterns in the donor cells than blastocysts produced via IVF. Oct4 was de-methylated in SCNT blastocysts, in apparent contrast to the methylation observed in IVF controls. In our study, Oct4 regulation was
not associated with any of the four histone modifications studied in any cell type analyzed. We should point out, however, that these four histone modifications represent a fraction of the documented modifications associated with epigenetic regulation. Various other histone modifications could play a role in the regulation of Oct4.

Trophoblast cells were analyzed in an effort to differentiate between embryonic chromatin from the ICM and the trophectoderm. The histone profiles observed in the trophoblast cells did not show any significant association with the genes in question, confirming that these three active genes are more closely associated with the ICM cells (Stice et al., 1996; Roberts et al., 2004; Bernstein et al., 2006). The immunofluorescence data presented here also confirms that these three TFs are primarily localized to the ICM of the blastocyst.

Other researchers have utilized ChIP assays to examine the epigenetic components of gene regulation for Nanog, Sox2, and Oct4. In mouse ES cells, Nanog and Sox2 loci have large regions of association with H3K4me3 (Mikkelsen et al., 2007). Another group examined the effect of acetylation of histone H3K9 and K27 on genes associated with regulating cell growth, immune response, and signal transduction in bovine cells (Shin et al., 2012). Sox2 and Nanog have also been shown to be overexpressed the ICM of bovine blastocysts, yet Oct4 expression did not differ significantly between ICM and trophoblast cells (Ozawa et al., 2012). Oct4 is also expressed by all cells in the mouse embryo between the 1-cell and late-blastocyst stage (Dietrich and Hiiragi, 2007; Plachta et al., 2011). Bovine fibroblast cells infected with retroviral Oct4 vectors and used in SCNT produced blastocysts with higher levels of histone H3K9 and K27 trimethylation than control blastocysts, yet Sox2 and Nanog
expression levels were not altered significantly in the SCNT blastocysts (Goissis et al., 2013).

_Nanog, Oct4, Sox2_, and other transcription factors, are associated with H3K4me3 and H3K27me3 in mouse ES cells, creating bivalent domains of activating and repressing histone modifications (Fouse et al., 2008). The global distributions of H3K4me3 and H3K9me2 have been compared between bovine fibroblasts, IVF, and SCNT embryos, yet both types of blastocyst-stage embryos showed no significant differences (Wu et al., 2011). Histone modification profiles are gene, tissue, and age specific, yet combinations have been shown to act cooperatively to prepare the chromatin for its transcriptional fate (Wang et al., 2008). Nevertheless, such differences in histone profiles associated with these pluripotent dependent genes in developing embryos were not anticipated. These differences in histone patterns add to the complexity of the events associated with nuclear reprogramming and the epigenetic mosaics associated with gene regulation.

The pattern of aberrant epigenetic regulation detected in bovine blastocysts, coupled with similar mRNA levels in two of the three genes in question, agrees with findings of other studies. In particular, global gene expression profiles in bovine SCNT embryos have been shown to differ significantly at the eight and sixteen cell stage (Smith et al., 2005; Aston et al., 2009). However, by the blastocyst stage, the gene expression differences between fertilized and cloned embryos had narrowed. This pattern suggests that more time during development is needed for the SCNT embryo to produce an expression profile similar to conventionally fertilized embryos. Aston et al. (2010) also determined that _Oct4_ expression in bovine IVF blastocysts was significantly different from SCNT blastocysts (Aston et al., 2010). The correlation found between the mRNA
levels and the protein expression is obviously self-validating, however post-translational modifications could play a role in rectifying the aberrant expression of Oct4 in SCNT embryos. The fact that these three TFs act in concert to regulate the expression of other key pluripotent factors, along with their role in controlling other downstream genes important to pluripotency, adds to the complexity of the regulatory network associated with embryonic stem cells (Pan and Thomson, 2007). Understandably, any deficiencies regarding any of the key-components in this pluripotent network should impact embryonic developmental success. Although Nanog and Sox2 expression levels are similar in SCNT and IVF embryos, aberrant Oct4 expression could markedly inhibit successful development due to its role in working synergistically with other important TFs.

These three genes represent important hallmarks associated with an embryonic stem-cell state. The data presented here pinpoints benchmarks relative to nuclear reprogramming and successful SCNT. The fact that a terminally differentiated fibroblast cell can be successfully reprogrammed and give rise to a competent embryo and subsequent offspring confirms the unique capacity of the components found within the unfertilized oocyte. Although deficiencies still exist, future studies will continue to provide insight into the epigenetic mechanisms involved in SCNT. A complete understanding and rectification of any aberrant mechanisms during development will yield exponential benefits to the fields of reproductive biology, cancer research, and stem cell biology.
MATERIALS AND METHODS

Oocyte Collection and Maturation

Bovine oocytes were collected and matured according to the published practices of our laboratory (Li et al., 2004). Specifically, bovine ovaries were collected from a local abattoir (EA Miller, Hyrum, UT) and cumulus oocyte complexes (coc) were aspirated from 3-8 mm follicles using an 18-gauge needle connected to a 50-ml centrifuge tube and a vacuum pump. Oocytes with multiple cumulus cell layers were washed four times in PB1⁺ (phosphate-buffered saline with Ca²⁺, Mg²⁺, 5.55 mM glucose, 0.32 mM sodium pyruvate, and 3mg/ml BSA). The oocytes were then transferred in groups of 50 to 500 µl of M199 with 10% FBS, 0.5 µg/ml FSH, 5 µg/ml LH, 100 µg/ml streptomycin, and 100 units/ml penicillin. Nunc 4-well IVF dishes (Nunc, Milwaukee, WI) were used as culture dishes, and the oocytes were incubated for 22-24 hr in 5% CO₂ at 39°C.

IVF Embryo Production

Following maturation, oocytes were fertilized with cryopreserved semen (Hoffman AI, Logan, UT). Live sperm was separated using a 45/90% percoll gradient, placed in fert-TALP and capacitated with a heparin concentration of 10 µg/ml (Reed et al., 1996). Diluted sperm was aliquoted to Nunc 4-well IVF dishes with groups of 50 mature oocytes in 500µl, and allowed to incubate for 18h in 5% CO₂ at 39°C. Following this incubation the oocytes were placed in 1 ml PB1⁺ and 10 mg/ml hyalurondase, vortexed for 2 min and 40 sec, washed in microdrops of PB1⁺ and cultured in CR2 media in groups of 50 (Rosenkrans and First, 1994). Culture media was supplemented with 200
μl of fresh CR2 every 48 hr, and day 7 blastocysts were used in groups of 10 according to the needed procedures.

**SCNT Embryo Production**

Following maturation, cumulus cells were removed as described above. Oocytes with a first polar body were selected. The first polar body and metaphase plate were enucleated, and single fibroblast cells were transferred into the perivitelline space of the enucleated oocytes. Fusions were done in mannitol fusion medium (Wells et al., 1999) by two electric DC pulses of 2.2 kV/cm for 25 μsec. Embryos were then held in CR2 medium for 1-2 hr prior to activation. Fused embryos were activated between 23 and 25 hr from the onset of maturation by exposure to 5 μM ionomycin for 5 min followed by 5 hr incubation in 10 μg/ml cyclohexamide. An equal number of blastocysts were produced via SCNT as were used in IVF control groups μChIP, RT PCR, and immunohistochemistry assays.

**μChIP Chromatin Preparation**

Each μChIP assay required chromatin from around 1,000 cells or 10 expanded blastocysts. This protocol was developed by Dahl and Collas, and adapted to accommodate DNA obtained from a low number of bovine embryos (Dahl and Collas, 2008). Briefly, a group of 10 blastocysts were selected and suspended in 500 μl PBS and 20mM Na-butyrate in a 0.6 ml tube at room temperature. Na-butyrate is used to avoid artefactual histone hyper-acetylation (Dahl and Collas 2008). Formaldehyde was added at 1% vol/vol final concentration to cross-link the DNA to the histone proteins located within 2 Angstroms of DNA. 57 μl of 1.25 M Glycine was used to quench the
formaldehyde at room temp for 5 min. Cells were centrifuged at 470 x g for 10 min at 4°C in a swing-out rotor. The supernatant was aspirated and discarded, leaving 30 μl of the solution with the pellet. The cells were then resuspended in 500 μl ice cold PBS/Na-butyrate by gentle vortexing and centrifuged again at 470 x g for 10 min at 4°C as before. This washing procedure was repeated twice. Upon aspiration of the last wash, about 20 μl of the PBS/Na-butyrate was left with the pellet.

After washing, 200 μl lysis buffer (50mM Tris-HCl pH to 8.0, 10 mM EDTA, 1% wt/vol SDS, 1 mM PMSF, 1 mM Protease Inhibitor, and 20 mM Na-butyrate) was added at room temperature, vortexed twice for 5 sec and put immediately on ice. PMSF, protease inhibitor, and Na-butyrate were added just before use to all working solutions. After at least 5 min on ice, the samples were sonicated using the 550 Sonic Dismembrator (Fisher Scientific) for 7 x 30 sec, with 2-min pauses on ice between each 30-sec session. With the 550 Sonicator, the cycle was set to 0.5 and intensity was set to 30% power. Sonication produced chromatin fragments of 500 base pairs. 100 μl RIPA ChIP buffer (10 mM Tris-HCl pH to 7.5, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% vol/vol Triton X-100, 0.1% wt/vol SDS, 0.1% wt/vol Na-deoxycholate, 1 mM protease inhibitor mix, 1mM PMSF, 20 mM Na-butyrate) was added following sonication, and mixed by vortexing. Samples were then centrifuged at 12,000 x g for 10 min at 4°C and the supernatant (chromatin) was aspirated and transferred to a 1.5 ml tube chilled on ice. Another 100 μl RIPA ChIP buffer was added to the remaining pellet and mixed by vortexing, followed by centrifugation at 12,000 x g for 10 min at 4°C and aspiration of the supernatant. This yielded 420 μl chromatin suitable immediately for ChIPs or stored at -80°C for up to 2 months (prolonged storage has not been tested).
µChIP Antibody-bead Complexes

A slurry of Dynabeads Protein A (Invitrogen, Carlsbad, CA) was prepared using 11.25 µl Dynabead stock solution/ChIP. Each assay included one ChIP as a negative control and one ChIP for each gene of interest. The total Dynabead solution was placed in a 0.6 ml-tube and placed in a magnetic rack where the beads were captured and the buffer was removed and discarded. The beads were then washed 3 times by adding 400 µl RIPA buffer (10 mM Tris-HCl pH to 7.5, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% vol/vol Triton X-100, 0.1% wt/vol SDS, 0.1% wt/vol Na-deoxycholate) vortexed, recaptured in the magnetic rack, and the RIPA discarded. After the final wash 11 µl RIPA/ChIP was added, the tube was vortexed and placed immediately on ice. An 8-tube-strip of 0.2 ml PCR tubes was used for subsequent steps to facilitate handling. One tube per ChIP was used, and each tube received 90 µl RIPA buffer, 10 µl washed Dynabeads, and 2.4 µg antibody. Negative controls received either no antibody or a preimmune antibody. The tube strip was then placed on a rotator at 40 r.p.m. for 2 hr at 4 °C. After incubation the antibody-bead complexes were captured using the magnetic rack, the RIPA was then removed, 100 µl prepared chromatin from above was added, the beads were released and mixed by gentle agitation and finally placed on a rotator at 40 r.p.m. for 2 hr at 4 °C. Following incubation, the tubes were centrifuged briefly in a mini centrifuge to bring down liquid trapped in the lids and placed on the magnetic rack. The beads and precipitated chromatin were then washed three times by gently aspirating and discarding the supernatant, adding 100 µl of ice-cold RIPA buffer, gently agitating and four minutes on a rotator at 40 r.p.m. at 4 °C. After the final wash and removal of the supernatant, 100 µl of TE buffer was added, followed by another 4 min on the rotator at
40 r.p.m. at 4 °C. The contents of each tube were transferred to a clean 0.2 ml tube chilled on ice, and the tubes were placed in the magnetic rack where the chromatin-bead complexes were captured again. The TE was subsequently removed, and 40 µl of Chelex-100 (Bio-Rad Laboratories, Hercules, CA) was added to each tube and vortexed for 10 s. 100 µl of prepared chromatin was also placed in a 0.6-ml tube as an input sample, and both input and μChIP samples were processed as outlined below.

**Purification of Input and Precipitated Chromatin**

To the input chromatin sample 10 µl acrylamide carrier and 250 µl of 96% ethanol at -20 °C were added. After thorough vortexing, the sample was placed at -80 °C for 30 min. The sample was then thawed and centrifuged at 20,000g for 15 min at 4 °C. Following centrifugation, the supernatant was aspirated and discarded, and the pellet was washed in 500 µl of 70% ethanol. To the dried pellet 40 µl of Chelex-100 was added and the sample was vortexed for 10 sec.

All samples containing Chelex-100 were boiled for 10 min and then cooled to room temperature. Proteinase K (1 µl, Qiagen, Valencia, CA) was added, and each tube was vortexed for 10 s and incubated at 55 °C at 1,300 r.p.m. for 30 min. The samples were then boiled again for 10 min and centrifuged for 10 sec. 30 µl was aspirated and transferred using a silicon tip to a clean 0.6 ml tube chilled on ice. 20 µl of molecular grade water was added, and each tube vortexed 2 x 10 sec and then centrifuged for 10 sec. Another 15 µl of supernatant was transferred to the corresponding 0.6 ml tube yielding 45 µl of precipitated DNA. Careful consideration was taken to ensure that no beads were aspirated and that each tube of purified DNA contained equal volumes to maintain consistency.
Quantitative PCR

SYBR Green real-time PCR (Abgene, Rochester, NY) was used to quantify the precipitated DNA. Each real-time PCR reaction was performed in duplicate on clear 96-well plates. Individual 25 μl real-time PCRs were prepared for all μChIP and input samples with each primer pair. Each reaction consisted of 12.5 μl Maxima™ SYBR Green 2X qPCR Master Mix (Fermentas, Glen Burnie, MD), both forward and reverse primers at 200 nM final concentration, 5 μl template DNA, and 5.5 μl molecular grade water. The same PCR program was used for each primer set: 15 min at 95 °C for activation of Maxima™ Hot Start Taq DNA polymerase, followed by 40 cycles of 95 °C for 30 sec, 60 °C for 20 sec, and 72 °C for 30 sec and data acquisition. A standard curve was generated for each primer set using genomic DNA prepared with the positive control, allowing Ct values to be converted to ng of DNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene, and primers (Table 1) were designed using Primer3 software.

Table 1. Primers used for μChIP PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sox2</strong></td>
<td>Forward</td>
<td>TTTCA CGTTTGCAACTGTCC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CATCACCCACAGCAAATGAC</td>
</tr>
<tr>
<td><strong>Nanog</strong></td>
<td>Forward</td>
<td>CCCTAGAGTTGGATGCTTCG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CATTGGACTGGATGGCTCTT</td>
</tr>
<tr>
<td><strong>Oct4</strong></td>
<td>Forward</td>
<td>GTTGTAGGCTTTGCAAGCTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCTCCAGGTTG CCTCCTCACT</td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td>Forward</td>
<td>CCAACGTGTCTCTGTTGGGATC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAGCTTGACAAAGTG GTCGTT</td>
</tr>
</tbody>
</table>
RNA Extraction and Reverse Transcription

SCNT and IVF 7-day blastocysts were pooled in groups of 10, and an RNA easy kit was used to extract total RNA (Qiagen, Valencia, CA). Embryos were snap-frozen and resuspended in beta-mercapto ethanol and RLT buffer. After following the Qiagen protocol, cDNA was produced using Superscript® III Reverse Transcriptase (Invitrogen, Carlsbad, CA). 50 ng of random primers were used, and this cDNA served as the template in RT-Q PCR. Each primer set (Table 2) followed the same PCR protocol: 95 °C for 10 min, and then 40 cycles of 95 °C for 30 sec, 60 °C for 20 sec, and 72 °C for 30 sec. Each 15 µl reaction contained ABgene SYBR Green Master Mix (Thermoscientific, Waltham, MA), 200 nM forward and reverse primers, and 2 µl of template.

Table 2. Primers used for gene expression analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanog</td>
<td>Forward</td>
<td>ATGTTTGAAGAAAGTTAYGTGTT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATACCATCTCTAACACACCTT</td>
</tr>
<tr>
<td>Oct4</td>
<td>Forward</td>
<td>GTTAGAGGTTAAGGTTAGTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CATCCCTCCACACAATCATAAACC</td>
</tr>
<tr>
<td>Sox2</td>
<td>Forward</td>
<td>TAAGAGAGTGGAAGGAATTTA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CACAAATACAAACACAAACAAA</td>
</tr>
</tbody>
</table>

Immunohistochemistry

Protein levels were analyzed using immunofluorescence. SCNT and IVF 7-day blastocysts were produced as described, and a Fixation/Permeabilization kit from eBioscience (eBioscience SanDiego, CA) was used for protein fixation, membrane permeabilization, blocking agent, and antibody diluent. Nunc 4-well IVF dishes with 500 µl of solution were used for all dilutions and incubations with no more than five
blastocysts per well. Embryos were fixed with a working solution of 1-part fixation solution and 3-parts of the supplied diluent and incubated @ 4°C for 1 hr in the dark. Embryos were then washed twice in a working solution of permeabilization buffer (1-part supplied permeabilization media, 9-parts H₂O, and 2% normal mouse serum). This permeabilization working solution was used for each subsequent step. A volume of 1 µl of primary antibody was added to 500 µl of permeabilization working solution and the embryos were incubated for 1 hr at room temperature in the dark. The anti-Nanog antibody was a mouse monoclonal antibody pre-labeled with Alexa Fluor® 488 (eBioscience, San Diego, CA), the anti-Oct4 was a mouse monoclonal IgG₂b, and the anti-Sox2 was a goat polyclonal IgG antibody (Santa Cruz Biotechnology, Dallas, TX). After primary antibody incubation the blastocysts were washed twice in permeabilization working solution for 5 min and incubated with 0.5 µl of the corresponding secondary antibody/fluorescent tag and 1 µl of 1,000 µg/ml Hoechst dye for 30 min at room temperature in the dark. Embryos that were stained with the pre-labeled anti-Nanog antibody did not necessitate secondary antibody incubation. Embryos were then mounted on a slide in 7 µl of Glycerin, covered with a cover slip with micro drops of petroleum jelly on the corners, and sealed under the cover slip using a border of Permount Mounting Media (Fischer Scientific, Waltham, MA). Slides were kept in the dark for at least 24 hr to dry before being placed on the microscope for image processing.

**Statistical Analysis**

**µChIP Q-PCR**

A standard curve was produced using genomic DNA and Ct values were converted to ng of DNA. These values were then compared to the input sample, and
expressed as a percentage of DNA captured versus the input control that received no antibody. A two-way ANOVA was used to compare the source of DNA (IVF, SCNT, donor cell, or trophoblast cell), within each gene. Bonferroni’s post-hoc test was further used, and \( P \)-values less than 0.05 were considered significant.

**Real-Time PCR**

\( \Delta \Delta C_t \) values were computed and used for gene expression analysis (Livak and Schmittgen, 2001). First, differing amounts of cDNA was accounted for by normalization to \( C_t \) values of the \( GAPDH \) housekeeping gene. This \( \Delta C_t \) is calculated for each cell type by subtracting the \( C_t \) value of the gene of interest from the \( GAPDH C_t \) value. Finally \( \Delta \Delta C_t \) values are calculated by subtracting the \( \Delta C_t \) value of the gene of interest from the IVF \( \Delta C_t \) value of the same gene of interest. Fold increase or decrease in expression levels was calculated by the formula \( 2^{-\Delta \Delta C_t} \), and those values were used in the Student’s t-test for pair-wise comparisons. Significance was considered \( P<0.05 \).

**Immunofluorescence**

Embryos were imaged individually on a Zeiss Axio Observer microscope (Zeiss, Gottingen, Germany). A 20x objective was used, and digital images acquired using a high-resolution AxioCam mRM digital camera. Exposure time was set for each channel or fluorescent tag and kept consistent for each embryo type. Embryo images were then analyzed using Axiovision software. Briefly, a perimeter was manually drawn around each embryo and the total area was computed. Images from each channel (DAPI, GFP, and Cy3) were then analyzed for the total area within the embryo that had positive fluorescence in the corresponding channel. DAPI, or nuclear staining, provided the total
area of the nuclei in the embryo. GFP and Cy3 values were then calculated as a percentage of the area stained by DAPI. Because each protein in question is a nuclear protein, this calculation provides an adequate representation of the number or percentage of nuclei that contain the protein of interest. Groups of 20 blastocysts, 10 IVF and 10 SCNT, were imaged for each protein of interest, and the percentages for each gene were averaged together. The student’s t-test again was used to test significance ($P<0.05$).
REFERENCES


Li GP, Bunch TD, White KL, Aston KI, Meerdo LN, Pate BJ, Sessions BR. 2004. Development, chromosomal composition, and cell allocation of bovine cloned...
blastocyst derived from chemically assisted enucleation and cultured in conditioned media. Molecular reproduction and development 68(2):189-197.


