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Apoptotic and Epigenetic Induction of Embryo Failure Following Somatic Cell Nuclear Transfer

Aaron Patrick Davis
Utah State University

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APOPTOTIC AND EPIGENETIC INDUCTION OF EMBRYO FAILURE FOLLOWING SOMATIC CELL NUCLEAR TRANSFER

by

Aaron Patrick Davis

A dissertation submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Animal, Dairy and Veterinary Science

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UTAH STATE UNIVERSITY Logan, Utah

2013
ABSTRACT

Apoptotic and Epigenetic Induction of Embryo Failure Following Somatic Cell Nuclear Transfer

by

Aaron Patrick Davis, Doctor of Philosophy
Utah State University, 2013

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

Somatic cell nuclear transfer (SCNT) is a useful tool for selective breeding, conservation, and production of transgenic animals. Despite the successful cloning of several species, high rates of embryo failure following SCNT prevent the wide-scale use of the technique. Embryos produced through cloning have a higher incidence of developmental arrest, decreased developmental potential, frequent implantation failures, and increased incidence of abortion. The objective of this dissertation research was to characterize the factors that lead to SCNT failures by examining epigenetic and apoptotic pathways that can negatively influence the development of cloned preimplantation embryos.

Aberrant genome reprogramming is generally considered to be a key factor in the failure of SCNT embryo development. Therefore, we used bisulfite pyrosequencing technology to compare DNA methylation patterns of several genes critical for embryonic
development (*POU5F1, NANOG, SOX2, and KLF4*) in SCNT and *in vitro* fertilized (IVF) blastocyst stage embryos. The methylation profiles obtained from these experiments indicate that methylation patterns of the *POU5F1* gene were undermethylated compared to IVF embryos, suggesting reprogramming did occur, but that the reduced methylation was inappropriate for the blastocyst stage. Furthermore, aberrant methylation profiles were detected for *SOX2* and *NANOG*, suggesting that problems of genome reprogramming following SCNT can be gene-specific or localized.

Because high rates of apoptosis are associated with failure of preimplantation embryos, we compared the activation of the P53-mediated apoptosis pathway in individual IVF and SCNT preimplantation embryos at multiple developmental stages. This pathway is activated in response to cell stress and genomic instability, and in response to the expression of genes associated with somatic cell reprogramming. Evidence from gene expression and immunohistochemistry analyses suggests that the P53 pathway is frequently active in SCNT embryos. Also, we detected expression of several factors known to induce apoptosis more frequently and at higher levels in SCNT embryos.

Collectively, the work presented here illuminates some of the molecular consequences of incomplete or inappropriate genome reprogramming in cloned embryos. The identification of these factors may lead to interventions that target the apoptosis pathway during preimplantation development and increase SCNT success rates.

(183 pages)
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The cloning of domestic species has tremendous potential, as the technology can be used in selective breeding, conservation, and the production of transgenic animals. The technique of cloning involves the transplant of DNA from a cell to a recipient gamete. Following transfer to a surrogate, the cloned embryo may successfully complete development to a live offspring. Despite intensive research, the success rate of cloning remains prohibitively low, and the potential benefits of cloning have not yet been realized. Embryos produced from cloning suffer from high rates of embryo degradation, implantation failure, and abortion. The goal of this dissertation research project was to determine the mechanisms that cause the high rates of embryo failure in clones.

In order for embryo development to occur properly following cloning, the donor cell genome must undergo epigenetic reprogramming, a process that resets the donor nuclei to an embryonic state. Epigenetic markings alter the expression of genes within a
cell, and are one factor that prevents cells from reverting back to a primordial state. As part of this research, we measured methylation of DNA, a type of epigenetic mark, to determine the level of reprogramming that occurs in cloned embryos. Our results demonstrate that cloned embryos have abnormalities in reprogramming following cloning for some important genes. This observation provides further support for the notion that the inefficiency of the cloning process may be a result of the inability of donor cells to readily reactivate genes that have been epigenetically silenced.

A second area of research into the causes of cloning failure was to examine the role of programmed cell death within cloned embryos. Every cell contains an innate defense system that triggers programmed cell death when the cell is under severe stress or has undergone irreparable DNA damage. We observed increased incidence of programmed cell death in cloned embryos that contributes to the high rates of developmental failure. These findings may potentially allow therapeutics targeted at preventing cell death, and thereby increase the success of cloning.
ACKNOWLEDGMENTS

I give thanks to my advisor, Dr. Ken White, as well as my committee members, Drs. Abby Benninghoff, Thomas Bunch, Gregory Podgorski, and Clay Isom. I also thank Drs. Qinggang Meng, Chris Davies, Lee Rickords, Aaron Thomas, and Heloisa Rutigliano for their frequent assistance. I recognize Dr. Ben Sessions, Dr. Ki Aston, Dr. Ammon Bayles, Kira Morgado, Bryce Osborne, and Eric Innes for their help throughout my time at Utah State.

This work was supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, under agreement No. 08-34526-19199 and 09-34526-19808. In addition, I would like to acknowledge the financial support of the Utah Agriculture Experiment Station.

I would like to give special thanks to my parents, Sam and Robin, for their continued support and my wife, Jessica, for being there with me all along the way.

Aaron Patrick Davis
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Representative images and evaluation of apoptosis in SCNT and IVF embryos
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### Abbreviations

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<td>Δ-Ct</td>
<td>Delta-Delta Ct</td>
</tr>
<tr>
<td>ΔCt</td>
<td>Delta Ct</td>
</tr>
<tr>
<td>ACADSB</td>
<td>acyl-CoA Dehydrogenase</td>
</tr>
<tr>
<td>ACTB</td>
<td>ß-actin</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2 associated X protein</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell CLL/lymphoma 2</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-phosphate-guanine</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>CX43</td>
<td>Gap junction protein, alpha 1</td>
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<tr>
<td>C-MYC</td>
<td>V-MYC myelocytomatosis viral</td>
</tr>
<tr>
<td>DHDDS</td>
<td>Dehydrodolichyl diphosphate synthase</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>ETS2</td>
<td>V-Ets erythroleukemia virus E25</td>
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<tr>
<td>ES Cells</td>
<td>Embryonic stem cells</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner cell mass</td>
</tr>
<tr>
<td>IGF2R</td>
<td>Insulin-like growth factor 2 receptor</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
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<tr>
<td>IKZF5</td>
<td>IKAROS family zinc finger 5 (Pegasus)</td>
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<tr>
<td>IVF</td>
<td>In vitro fertilization</td>
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<tr>
<td>KLF4</td>
<td>Kruppel-like factor 4</td>
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<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
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<td>LIN28</td>
<td>Lin-28 homolog A</td>
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<td>MAN2A1</td>
<td>Mannosidase, alpha, class 2A, member 1</td>
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<td>NANOG</td>
<td>Nanog homeobox</td>
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<td>P21WAF1/Cip1</td>
<td>Cyclin-dependent kinase inhibitor 1A</td>
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<tr>
<td>P53</td>
<td>Tumor protein P53</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>Pe-G/trx-G</td>
<td>Polycomb/trithorax group complex</td>
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<td>PEG3</td>
<td>Paternally expressed 3</td>
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<td>POU5F1</td>
<td>POU class 5 homeobox 1</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SCNT</td>
<td>Somatic cell nuclear transfer</td>
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SOX2  
Sex determining region-box 2

TUNEL  
Terminal deoxynucleotidyl transferase dUTP nick end labeling

Symbols

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<tr>
<td>C</td>
<td>Celsius</td>
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<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>DC</td>
<td>Direct current</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>g</td>
<td>Gravity</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>mg</td>
<td>Milligrams</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>µsec</td>
<td>Microsecond</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
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<tr>
<td>sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>µg</td>
<td>Micrograms</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
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<tr>
<td>µM</td>
<td>Micromolar</td>
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CHAPTER 1
REVIEW OF LITERATURE

History of Somatic Cell Nuclear Transfer

The technique of somatic cell nuclear transfer (SCNT) was originally conceived in the early twentieth century by developmental biologist Hans Spemann, who sought to understand the process whereby somatic cells specialize during development. During this time he proposed an experiment in which a somatic cell would be transferred to an enucleated oocyte in order to determine the developmental potential of somatic cells. Although he lacked the appropriate instrumentation to carry out such an experiment, Spemann is credited with introducing the experimental design now known as SCNT.

Prior to Spemann’s proposed nuclear transfer experiment, Hans Driesch had demonstrated that each cell of an embryo contained the entire complement of genes necessary to carry out development. He accomplished this by separating individual blastomeres from developing sea urchin embryos and observed that each blastomere had the capacity to develop into a separate embryo (Spemann, 1938). This evidence was contrary to a competing hypothesis specifying that genes were segregated during development, and that the unique complement of genes found in an individual cell resulted in the unique phenotype of the somatic population. Using frog embryos, Spemann carried out a similar set of experiments in which early-stage embryos were artificially split resulting in two identical embryos, each capable of carrying out development (Spemann, 1938). Collectively, the work of Driesch and Spemann affirmed the hypothesis of complete genome replication, although their experiments were
restricted to early-stage blastomeres. The hypothesis that somatic cells lost genes during development could not be directly tested for several years until the instrumentation required for the nuclear transfer experiment originally devised by Spemann was developed.

The first experiment to use nuclear transfer as a technique was completed in 1952. The North American leopard frog (Rana pipens) was utilized for nuclear transfer using late blastula cells as a donor cell source (Briggs and King, 1952). The successful cloning of a frog affirmed the concept of nuclear equivalence, showing that genetic material was not lost during development and demonstrating that a differentiated nucleus could return to an undifferentiated state. Briggs and King eventually expanded their work to include different donor cell types to determine whether or not all cells have the same potential for dedifferentiation (King and Briggs, 1955). Further experiments showed that cloning efficiency declined in proportion to the degree of differentiation. Although the success rate was low, late gastrula cells could be used as a donor source to successfully generate tadpoles (Briggs and King, 1960). Building on these studies, Gurdon demonstrated that epithelial cells derived from early Xenopus tadpoles could be used as a source of donor cells to generate clones (Gurdon, 1962). In a later study, Gurdon successfully used keratinocytes from adult Xenopus as donor cells (Gurdon et al., 1975). While these experiments produced tadpoles, neither group was successful in generating adult frogs from cloning experiments.

In 1975, Bromhall was the first to apply the cloning procedure to mammals. Using rabbits, he observed that cell divisions could occur following nuclear transfer,
although development never continued beyond early cleavage stages (Bromhall, 1975). Using enucleated mouse zygotes as recipients for zygotic nuclei, McGrath and Solter generated mouse offspring that developed completely to term (McGrath and Solter, 1983). However, subsequent cloning attempts that used mouse blastomeres as a donor source failed to generate embryos that could initiate development (McGrath and Solter, 1984). Eventually, the cloning of domestic species using blastomeres as donor cells resulted in the birth of clones (Willadsen, 1986; Prather et al., 1987; Prather et al., 1989). Each successful cloning attempt used enucleated oocytes as donor cell recipients, rather than zygotes which had failed to produce clones in mice (McGrath and Solter, 1984). Willadsen successfully generated cloned cattle using blastomeres as donor cells (Willadsen et al., 1991), and Sims and First successfully cloned four calves from inner cell mass (ICM) cells that were briefly cultured in vitro prior to use as a donor cell (Sims and First, 1994).

The work of Sims and First established the use of cultured cells in SCNT procedures to generate mammalian offspring, but the benchmark of generating a cloned offspring derived from a somatic cell had yet to be achieved. Following several failed attempts, it was unknown if somatic nuclei were capable of being reprogrammed to an undifferentiated state.

In 1996, a research team produced a sheep cloned from the mammary epithelial cell of an adult sheep (Wilmut et al., 1997). Dolly, the offspring of these experiments, represented a major scientific breakthrough, as the cloned ewe was the first mammalian offspring produced from a somatic cell. Her birth indicated that somatic cells were, in
fact, capable of dedifferentiation to a totipotent state and could sustain embryo
development following transfer to an oocyte. Since the birth of Dolly, several somatic
cell lines have been used for SCNT in mice, including terminally differentiated cells
(Wakayama et al., 1998; Hochedlinger and Jaenisch, 2002).

To date, many species have been cloned from embryonic and/or somatic cell
lines, including: sheep (Wilmut et al., 1997), cattle (Cibelli et al., 1998a; Kato et al.,
1998; Wells et al., 1999; Kato et al., 2000), goat (Baguisi et al., 1999; Keefer et al.,
2001), pig (Betthauser et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000), mouse
(Wakayama et al., 1998; Wakayama and Yanagimachi, 1999; Hosaka et al., 2000),
monkey (Mitalipov et al., 2002), gaur (Lanza et al., 2000b), mouflon (Loi et al., 2001),
cat (Shin et al., 2002; Gomez et al., 2004), rabbit (Chesne et al., 2002; Challah-Jacques et
al., 2003), zebrafish (Lee et al., 2002), rat (Zhou et al., 2003), mule (Woods et al., 2003),
horse (Galli et al., 2003; Hinrichs et al., 2006), water buffalo (Suteevun et al., 2006), dog
(Lee et al., 2005), banteng (Sansinena et al., 2005), ferret (Li et al., 2006), deer (Berg et
al., 2007), pyrenean ibex (Folch et al., 2009), and camel (Wani et al., 2010).
Additionally, human embryos have been produced by SCNT, but have not been allowed
to develop past the blastocyst stage (Hwang et al., 2004; Tachibana et al., 2013).

All SCNT experiments have used variations of the first procedure established by
Briggs and King. Oocyte enucleation is achieved through the removal of the pronucleus
along with the metaphase spindle. Briggs and King performed transfer of the donor
nucleus by aspirating the blastomere into a narrow micropipette that ensured the cell
membrane was sheared, while leaving the nucleus intact, and then microinjecting the
nucleus into the oocyte (Briggs and King, 1952). Amphibian eggs do not require any form of oocyte activation beyond microinjection.

The SCNT technique is similar for mammals and amphibians, although cell fusion via electrofusion is preferred to microinjection for introduction of the donor cell to the mammalian recipient oocyte. However, microinjection of the donor cell nucleus remains the standard practice in the mouse, as murine oocytes tend to self-activate upon stimulation with an electric pulse (Wakayama et al., 1998). For the electrofusion procedure, the zona pellucida and oocyte membrane are pierced, and the first polar body, pronuclei and metaphase plate are removed. The donor cell, which is similar in size to the polar body, is then inserted between the oocyte membrane and zona pellucida. Electrofusion is used to fuse the two membranes. The cloned embryo is briefly cultured in vitro, then transferred to a surrogate for fetal development. In procedures using bovine oocytes, activation is initiated using the calcium ionophore compound ionomycin along with cycloheximide to briefly suspend protein synthesis.

Cloning has been a valuable tool for advancing science in several areas of biology including nuclear differentiation, nuclear reprogramming, genomic imprinting, and cellular aging. Many researchers have taken advantage of genetic engineering techniques to manipulate the donor cell genome and subsequently perform SCNT to produce transgenic offspring. One such example was the generation of a transgenic sheep that produced human clotting factor IX in its milk as a pharmaceutical for treatment of hemophilia (Schnieke et al., 1997). Cloning has been used to generate transgenic animals by random gene addition (Schnieke et al., 1997), gene knockout (McCreath et al., 2000),
and gene mutation (Rogers et al., 2008). Aside from the production of human proteins in transgenic cloned animals, SCNT also provides researchers the opportunity to modify an animal’s genome to reproduce desired genetic traits, such as increased casein content in the milk of transgenic cattle (Brophy et al., 2003) or the removal of antigens for potential xenotransplantation (Lai et al., 2002; Phelps et al., 2003). Transgenic clones can also be used for research into human disease (Denning et al., 2001).

Cloning can also be applied for animal reproduction. SCNT can be used to reproduce genetically superior animals as well as for the preservation of endangered species. Some endangered species have been cloned for this purpose, such as gaur (Lanza et al., 2000b), mouflon (Loi et al., 2001), banteng (Sansinena et al., 2005), and water buffalo (Suteevun et al., 2006). This procedure has also been used to clone an extinct species. The pyrenean ibex, a species that went extinct in 2000, was cloned in 2009 using cells frozen from one of the last remaining survivors (Folch et al., 2009). Even though the cloned animal survived throughout gestation, it died shortly after birth.

Cloning has also been applied in therapeutic applications including the generation of embryonic stem (ES) cells (Rideout et al., 2002; Barberi et al., 2003). ES cells have been derived from clones in mouse (Munsie et al., 2000; Wakayama, 2003) and human (Hwang et al., 2004; Tachibana et al., 2013), and ES-like cells have been generated in cattle (Cibelli et al., 1998b) and rabbit (Fang et al., 2006). Additionally, cloning could be used for pharmacology purposes, such as generating identical individuals for drug testing, although this potential has yet to be realized.
SCNT has also contributed to advances in basic biology. Some examples include cloning for the study of allelic regulation (Gerdes and Wabl, 2004), the study of secondary rearrangements of the Immunoglobulin genes (Koralov et al., 2005), and the determination that olfactory receptor neurons do not generate variation through gene rearrangements (Eggan et al., 2004; Li et al., 2004).

**Limitations in Generating Cloned Offspring**

The largest barrier to cloning is the high loss of cloned embryos during gestation. Most pregnancy failures occur early after implantation during the period critical to placental establishment (Heyman et al., 2002). Across most species, the success rate of reconstructed embryos that result in the birth of healthy offspring is less than 3% (Thibault, 2003). Reported success rates of various donor cells reported in Table 1-1. Cloned embryos that successfully implant regularly develop placental abnormalities (Yang et al., 2007). In mice, several problems associated with placental formation have been observed in SCNT pups (Wakayama and Yanagimachi, 1999; Tanaka et al., 2001; Jouneau et al., 2006; Wakisaka-Saito et al., 2006; Wakisaka et al., 2008) and similar observations have been made in cattle (Chavatte-Palmer et al., 2012). An oversized and dysfunctional placenta is frequently observed in both aborted clones as well as those that have survived to term. This abnormal placenta likely contributes to the high rates of fetal loss (Wakayama et al., 1998; Wakayama and Yanagimachi, 1999; De Sousa et al., 2001).

SCNT animals often die shortly after birth, with the most common cause of neonatal death thought to be respiratory distress and problems associated with circulation (Wilmut et al., 1997; Young et al., 1998; Hill et al., 1999). Placental deficiencies,
Table 1-1. Somatic Cell Nuclear Transfer Success Rates for Live Offspring in Various Species

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell Type</th>
<th>Success Rate (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>Adult Mammary</td>
<td>3.4</td>
<td>(Wilmut et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>Fetal Fibroblasts</td>
<td>5 to 20</td>
<td>(Schnieke et al., 1997)</td>
</tr>
<tr>
<td>Cattle</td>
<td>Fibroblasts</td>
<td>9 to 20</td>
<td>(Wells et al., 1999; Kato et al., 2000; Brophy et al., 2003)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Cumulus Cells</td>
<td>1 to 3</td>
<td>(Wakayama et al., 2000; Wakayama et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Sertoli Cells</td>
<td>6</td>
<td>(Ogura et al., 2000; Wakayama et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Natural Killer T-Cells</td>
<td>1 to 2</td>
<td>(Inoue et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>B and T Cells</td>
<td>&lt;1</td>
<td>(Hochedlinger and Jaenisch, 2002; Eggan et al., 2004; Li et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Neurons</td>
<td>&lt;1</td>
<td>(Eggan et al., 2004; Li et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Fibroblasts</td>
<td>1</td>
<td>(Wakayama and Yanagimachi, 1999; Wakayama et al., 2005)</td>
</tr>
<tr>
<td>Goat</td>
<td>Fetal Fibroblasts</td>
<td>3 to 10</td>
<td>(Baguii et al., 1999; Keefer et al., 2001)</td>
</tr>
<tr>
<td>Pigs</td>
<td>Granulosa Cells</td>
<td>1.2</td>
<td>(Polejaeva et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Fetal Fibroblasts</td>
<td>4</td>
<td>(Lai et al., 2002)</td>
</tr>
<tr>
<td>Cat</td>
<td>Fibroblasts</td>
<td>1.1</td>
<td>(Shin et al., 2002)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Cumulus Cells</td>
<td>1.6</td>
<td>(Chesne et al., 2002)</td>
</tr>
<tr>
<td>Rat</td>
<td>Fetal Fibroblasts</td>
<td>1.7</td>
<td>(Zhou et al., 2003)</td>
</tr>
<tr>
<td>Horse</td>
<td>Fibroblasts</td>
<td>5.9</td>
<td>(Galli et al., 2003)</td>
</tr>
<tr>
<td>Dog</td>
<td>Fibroblasts</td>
<td>0.2</td>
<td>(Lee et al., 2005)</td>
</tr>
<tr>
<td>Ferret</td>
<td>Cumulus Cells</td>
<td>1.8</td>
<td>(Li et al., 2006)</td>
</tr>
</tbody>
</table>
neonatal edema, urogenital tract defects, and problems with the respiratory and cardiovascular systems are typical abnormalities in cloned offspring that survive to term (Hill et al., 1999; Renard et al., 1999; Hill et al., 2000a; De Sousa et al., 2001; Chavatte-Palmer et al., 2002). Reduced immunity, kidney malformation (Lanza et al., 2000a; McCreath et al., 2000), predisposition to infection, immature lungs, and general weakness (Zakhartchenko et al., 2001) are also commonly observed in cloned neonates. In addition, clones often suffer from a collection of growth malformations known as large offspring syndrome, which is characterized by large size and a general failure to thrive (Young et al., 1998).

SCNT offspring have a much higher incidence of abnormal phenotypes than offspring produced in vivo or by in vitro fertilization (IVF) (Han et al., 2003; Thibault, 2003). The abnormal phenotypes are generally held to be the result of incomplete epigenetic reprogramming of the somatic cell genome after introduction into the oocyte (Wilmut et al., 1998; Colman, 1999; Kikyo and Wolffe, 2000; Solter, 2000; Dean et al., 2001; Humpherys et al., 2001; Reik et al., 2001; Niemann et al., 2008).

The Epigenetic Landscape

Epigenetics is defined as heritable changes in gene expression that are not due to changes to the DNA sequence. Epigenetic factors include DNA methylation, histone modifications, and polycomb-trithorax complex, all of which modify the physical characteristics of chromatin, and lock transcriptional regions in either an active or silent expression state. Epigenetic signals are stably inherited after cell division and enable daughter cells to recapitulate the transcriptional program of the parent cell. The
development of multicellular organisms requires a specific transcriptional program of
gene expression for each individual cell type. In animals, over 50% of genes are inactive
in any given cell (Bergman and Cedar, 2013) The unique epigenetic program of each cell
maintains both the long-term silencing of inappropriate genes and the active transcription
of genes that are necessary to carry out the functions of the cell. Epigenetic patterns are
established during embryogenesis and early development and are maintained within adult
somatic cells.

Epigenetic memory is maintained through methylation of DNA. Once established,
the pattern of methylation can be copied in each cell generation to maintain a long-term
epigenetic signal. Vertebrates have the highest levels of DNA methylation in the animal
kingdom and contain methylated regions dispersed throughout most of their genomes
(Bird, 2002). The widespread distribution of DNA methylation patterns suggests
methylation is involved in multiple aspects of epigenetic control (Tweedie et al., 1999).
Researchers have postulated that DNA methylation originated as a genomic defense
system that allowed a cell to inactivate viral and transposable elements inserted into DNA
(Liu et al., 1994; Woodcock et al., 1997; Yoder et al., 1997; Walsh et al., 1998). DNA
methylation has evolved to play major roles in X-chromosome inactivation (Mohandas et
al., 1981; Csankovszki et al., 2001), genomic imprinting (Bartolomei et al., 1993; Li et
al., 1993; Stoger et al., 1993), the maintenance of chromosome stability (Chen et al.,
1998; Moarefi and Chedin, 2011), and possibly in RNA splicing variation (Schwartz et
al., 2009; Laurent et al., 2010; Shukla et al., 2011). Transcription of genes on the
inactive X-chromosome is induced within cells treated with agents that cause global
demethylation (Mohandas et al., 1981; Graves, 1982; Venolia et al., 1982). Induction of demethylation also causes the aberrant transcription of retroviral elements normally dormant within the genome (Stewart et al., 1982; Jaenisch et al., 1985).

DNA methylation is also involved in the long-term silencing of tissue-specific genes, as methylation of the transcriptional start site is associated with transcriptional inactivation of genes (De Smet et al., 1996; De Smet et al., 1999; Jaenisch and Bird, 2003; Shiota, 2004; Han et al., 2011; You et al., 2011). Embryos and cultured cells with reduced methylation aberrantly express a number of genes that are normally repressed (Stancheva and Meehan, 2000; Jackson-Grusby et al., 2001). Some tissue-specific genes maintain methylation throughout development and are correspondingly transcriptionally inactive until a demethylation event relieves inhibition to allow gene expression (Han et al., 2011). Although DNA methylation is an important mechanism for long-term silencing of tissue-specific genes, less than 10% of genes are regulated by DNA methylation (Illingworth and Bird, 2009). Multiple tissue-specific genes undergo long-term transcriptional inactivation without changes to DNA methylation patterns (McKeon et al., 1982; Bird, 1987). However, some genes require the presence of methylation for long-term gene inactivation (Venolia and Gartler, 1983; Kass et al., 1997; Hashimshony et al., 2003).

In mammals, DNA methylation occurs almost exclusively on cytosine nucleotides in the context of CpG dinucleotides. Approximately 75% of CpG dinucleotides are methylated (Ehrlich et al., 1982), most of which are concentrated in well-defined regions. However, any CpG dinucleotide can be methylated at any time under special
circumstances or in abnormal cell types, such as in cancer cells (Bird, 2002). Approximately 98% of CpG dinucleotides are dispersed throughout the genome at low density and are highly methylated. The remaining 2% of CpG dinucleotides are in densely packed regions consisting of approximately one CpG dinucleotide per ten base pairs. These dense clusters of CpG dinucleotides are referred to as CpG islands, and are generally void of methylation (Brandeis et al., 1994; Straussman et al., 2009).

CpG islands are a unique feature of vertebrates and are defined as regions of DNA with a high density of CpG dinucleotides (Illingworth and Bird, 2009). These islands are associated with approximately 60% of genes and are commonly found at the 5’-end of genes (Antequera and Bird, 1993). CpG islands are often associated with transcription factor binding regions, and methylation of these regions is linked to the transcriptional regulation of the associated gene (Bird, 1986; Bird, 1987; Antequera and Bird, 1993; Cuadrado et al., 2001; Bird, 2002; Illingworth et al., 2008).

Repression by DNA methylation may be weak or strong depending on the methylation density (Boyes and Bird, 1992; Hsieh, 1994). Methylation of DNA leads to inhibition of gene expression via one of two mechanisms. The first involves the direct blocking of transcription factor binding to DNA by the methyl groups attached to CpG dinucleotides (Prendergast and Ziff, 1991; Bell and Felsenfeld, 2000; Hark et al., 2000; Szabo et al., 2000; Holmgren et al., 2001). The second, and likely more prevalent mode of gene silencing, involves proteins that bind selectively to methylated DNA and either block transcription factor access to DNA, or recruit chromatin condensation complexes that silence the chromatin region (Meehan et al., 1989; Jones et al., 1998; Nan et al.,
Several proteins have been discovered that selectively bind methylated DNA (Nan et al., 1993; Cross et al., 1997; Nan et al., 1997; Hendrich and Bird, 1998). One such protein, KAISO, has been found to initiate methylation-dependent repression upon binding to methylated DNA (Prokhortchouk et al., 2001). Furthermore, histone deacetylases can be recruited to methylated DNA in order to further reinforce repression (Wade and Wolffe, 2001; Lin et al., 2007).

The family of DNA methyltransferases (DNMT) (Bestor, 1992; Miniou et al., 1994; Okano et al., 1998a,b; Pradhan et al., 1999; Hansen et al., 2000; Kondo et al., 2000; Rhee et al., 2000) is responsible for establishing and maintaining patterns of DNA methylation. DNMTs are essential for normal development (Li et al., 1992; Okano et al., 1999). Both mice and humans with deficiencies in DNMT lack methylation on the inactive X-chromosome and pericentromeric repetitive DNA sequences and express genes that are normally inactive (Bestor, 2000; Sado et al., 2000; Stancheva and Meehan, 2000).

During DNA replication, DNMTs associate with the replication machinery to duplicate methylation patterns in newly synthesized DNA (Leonhardt et al., 1992). In addition to DNMTs, methylation requires a number of accessory proteins that likely give DNMTs access to heterochromatic regions (Gibbons et al., 2000; Dennis et al., 2001). The de novo establishment of DNA methylation can be targeted to CpG islands by a RNA-directed mechanism (Wassenegger et al., 1994; Bender, 2001; Matzke et al., 2001). DNMTs are also attracted to specific genomic regions (Turker, 1999) by modified histone H3 proteins in chromatin (Tamaru and Selker, 2001), and by histone modifying
complexes that recruit DNMTs to condensed regions (Feldman et al., 2006; Epsztejn-Litman et al., 2008).

DNA methylation does not directly stop gene transcription in actively expressed genes, but rather acts as a secondary mechanism that locks in a transcriptionally silent state after the region has been rendered transcriptionally inactive by some other mechanism (Gautsch and Wilson, 1983; Niwa et al., 1983; Lock et al., 1987; Keohane et al., 1996; Pannell et al., 2000; Wutz and Jaenisch, 2000). Transgenes that contain a CpG island can maintain active transcription following genome insertion. However, in the event that promoter function is impaired, DNA methylation will accumulate upon the transgene and prevent future expression (Macleod et al., 1994). Aberrant de novo methylation, such that occurs in cancer, primarily accumulates on regions after they have become transcriptionally inactive. This reinforces the view of DNA methylation as a ‘lock’ rather than a trigger (Ohm et al., 2007; Widschwendter et al., 2007; Gal-Yam et al., 2008). However, the role played by DNA methylation in maintaining silencing is essential for eliminating plasticity and preventing a cell from reverting back to a less differentiated state (Wareham et al., 1987; Epsztejn-Litman et al., 2008).

The distribution and extent of DNA methylation varies among species. *Caenorhabditis elegans* lacks detectible cytosine methylation or DNMT expression (Bird, 2002). *Drosophila melanogaster* contains a DNA methyltransferase (Hung et al., 1999; Tweedie et al., 1999) and maintains low levels of DNA methylation, primarily in CpT dinucleotides (Gowher et al., 2000; Lyko et al., 2000). Both of these species maintain epigenetic memory predominantly through the Polycomb/Trithorax Group Complex (Pc-
G/trx-G) (Paro et al., 1998; Pirrotta, 1999; Francis and Kingston, 2001). Pc-G/trx-G is a multiprotein complex that establishes a transcriptional state at targeted genomic regions and stably maintains either an active or repressive state of chromatin throughout development. The Pc-G/trx-G achieves a similar outcome as DNA methylation in mammals (Yoder et al., 1997; Birchler et al., 2000). Although mammals also utilize the Pc-G/trx-G system, they appear to have evolved a more complex use of DNA methylation that is utilized in conjunction with the Pc-G/trx-G system (Sado et al., 2000; Wang et al., 2001).

**DNA Methylation and Somatic Cell Nuclear Transfer**

Modest improvements to cloning efficiencies have been achieved by manipulating factors such as SCNT activation procedure, donor cell passage number, culture conditions, and cell cycle stage. Changes in these factors have been shown to alter gene expression in SCNT embryos (Wrenzycki et al., 2001). However, developmental success still remains diminished compared to *in vivo* and IVF-produced embryos, likely because the problems associated with clones are epigenetic in nature.

DNA methylation establishes chromatin structure required for successful development (Hashimshony et al., 2003). Failure to maintain a suitable methylation program leads to multiple developmental abnormalities, as embryos deficient in DNA methylation can either fail to develop or abort during early gestation (Bestor, 2000; Bird, 2002). The establishment of DNA methylation patterns is a complex, coordinated process that occurs progressively throughout development. Following fertilization, the paternal pronucleus undergoes active demethylation that occurs by enzymatic removal of
methyl groups from CpG sites. This process occurs exclusively on the paternal genome and is completed prior to the DNA replication at the first cleavage division (Mayer et al., 2000; Oswald et al., 2000). The maternal pronucleus does not undergo active demethylation. Instead, DNA methylation levels are gradually diluted through cleavage divisions, due to inhibition of methylation on the nascent DNA strand (Reik et al., 2001; Santos et al., 2002). The result is global demethylation during preimplantation development during which methylation levels drop approximately 30% following fertilization (Monk et al., 1987; Kafri et al., 1992). Demethylation as part of embryonic development likely occurs to remove any methylation marks imposed during gametogenesis (Smith et al., 2012) as well as to remove methylation patterns from developmentally significant genes that may be methylated in the sperm (Farthing et al., 2008). By the time the embryo develops to the morula stage, methylation is primarily found in imprinted genes and repetitive elements (Sanford et al., 1987; Walsh et al., 1998; Reik and Walter, 2001). However, upon cellular differentiation at the blastocyst stage, new methylation patterns begin to be established, and clear differences in methylation signature can be seen between the trophectoderm and inner cell mass (ICM) (Turker, 1999; Santos et al., 2002).

A major challenge in reprogramming a somatic cell during SCNT is that reprogramming of donor cell methylation patterns must recapitulate the removal and reestablishment of methylation patterns that occur in normal embryos. Clones must epigenetically reset genes required for embryo development prior to the maternal-to-embryo transition, after which developmental progress is dependent on the transcriptional
program of the donor cell genome. Several studies have observed abnormal levels of DNA methylation in cloned embryos (Dean et al., 1998; Dean et al., 2001; Kang et al., 2001; Ohgane et al., 2001; Kang et al., 2002). The mechanism responsible for the abnormalities in methylation patterns following SCNT is not clear, but it is likely that the donor cell genome responds differently to the oocyte cytoplasm than the pronucleus.

Examples of methylation defects in clones include errors in X-chromosome inactivation (Keohane et al., 1996; Eggan et al., 2000; Xue et al., 2002; Senda et al., 2004; Nolen et al., 2005), irregularities in the methylation patterns of imprinted genes and their expression patterns (Humpherys et al., 2001; Inoue et al., 2002; Mann et al., 2003), dysregulation of DNMTs (Chung et al., 2003), and overall abnormalities in patterns of DNA methylation (Bourc'his et al., 2001; Dean et al., 2001; Humpherys et al., 2001; Kang et al., 2001).

Analysis of the organs of cloned neonate mice reveals that a large number of genes are aberrantly methylated (Ohgane et al., 2001; Humpherys et al., 2002). In addition to alterations in DNA methylation, clones exhibit unusual patterns of histone acetylation and methylation (Santos et al., 2003), alterations in gene expression (Humpherys et al., 2002), and failure to transcribe key developmental genes (Boiani et al., 2002; Boiani et al., 2003). These abnormalities reduce the developmental potential of clones and likely terminate the development of many cloned embryos prior to implantation.

Imprinted genes are established by methylation marks that are placed on genes during late stages of gametogenesis that are specific to the parent of origin (Constancia et
al., 1998; Tilghman, 1999). Once disrupted, imprinting patterns cannot be reestablished unless cells pass through germ line (Tucker et al., 1996) as imprinted patterns are removed in the germ cell line and then reset prior to gametogenesis (Tada et al., 1998). Clones display abnormalities in most imprinted genes, and the disruption of imprints is linked to the overgrowth commonly seen in clones (Humpherys et al., 2001). The large offspring syndrome seen in clones could be a result of abnormal patterns of imprinting (Constancia et al., 1998; Tilghman, 1999). Similar overgrowth abnormalities have been observed in humans that harbor defects in imprinted genes, as well as in mice in which targeted mutagenesis has altered the expression of imprinted genes (Jaenisch, 1997).

Aberrations in DNA methylation also contribute to placental abnormalities commonly observed in clones. Deficient placental formation has been characterized in SCNT sheep (De Sousa et al., 2001), cattle (Hill et al., 2000a; Chavatte-Palmer et al., 2002), and mice (Wakayama and Yanagimachi, 1999; Tanaka et al., 2001). Perinatal problems have also been observed in clones (Hill et al., 1999; Chavatte-Palmer et al., 2002; Pace et al., 2002), and these may be the result of poor placental development. In normal embryos, the trophoectoderm is globally undermethylated (Monk et al., 1987; Santos et al., 2002). However, in clones this pattern is reversed, with the trophoectoderm being highly methylated globally (Bourc'his et al., 2001; Dean et al., 2001; Kang et al., 2001; Kang et al., 2002). The abnormal methylation and gene expression patterns in the trophoectoderm of clones likely contribute to abnormal placental development.

Interestingly, the epigenetic signature of cloned embryos deviates not only from IVF and in vivo control embryos, but also differ significantly among cloned embryos
(Dean et al., 2003; Jouneau and Renard, 2003; Kang et al., 2003; Santos et al., 2003; Hochedlinger et al., 2004). This observation suggests that methylation patterns following SCNT are established stochastically. The random patterns of epigenetic marks most likely contribute to the aberrant patterns of gene expression (Ohgane et al., 2001; Humpherys et al., 2002).

Further evidence that cloning failure is largely epigenetic in nature is that the types of epigenetic abnormalities in cloned species, at least among farm animals, are not seen in the offspring of clones (Tamashiro et al., 2002; Jaenisch, 2004). Offspring born to clones are normal and do not exhibit any of the developmental abnormalities or high abortion rates associated with clones, indicating that one generation is sufficient to reestablish normal epigenetic architecture (Zhang et al., 2004).

**Epigenetic Reprogramming**

Developmental failure of SCNT embryos is thought to be the result of incomplete epigenetic reprogramming following transfer of the somatic cell (Wilmut et al., 1998; Colman, 1999; Kikyo and Wolffe, 2000; Solter, 2000; Dean et al., 2001; Humpherys et al., 2001; Reik et al., 2001; Niemann et al., 2008). Nuclear reprogramming refers to transcriptional and epigenetic modifications required to achieve totipotency. The desired outcome of nuclear transfer is for the somatic nucleus to achieve the same transcriptional pattern as a zygotic nucleus required to successfully direct embryogenesis. This process requires silencing of genes expressed in somatic cells, and expression of zygotic-specific genes.
As part of normal mammalian development, several major reprogramming events alter the genomic state and clear the genome of epigenetic markings as a prelude to development. The first reprogramming event occurs during primordial germ cell formation in which imprinted genes have their methylation signatures removed, and differentiation is reestablished to a totipotent state as part of gametogenesis (Tada et al., 1997; Reik and Walter, 2001). A second reprogramming event occurs upon fertilization when gamete-specific marks are erased. In addition to reprogramming associated with development, a partial reprogramming event is associated with cancer development, in which cells undergo partial dedifferentiation and the epigenetic signature is altered in ways that accommodate tumor development and growth. Reprogramming can also be artificially induced via ectopic expression of key reprogramming factors in order to generate induced pluripotent stem cells (iPSC) (Takahashi et al., 2007; Yu et al., 2007).

Reprogramming during preimplantation development is likely a dynamic process. Rather than setting the donor cell nuclei to a ‘zero’ state, reprogramming appears to continue throughout embryo development and potentially beyond. The demethylation event common to both the paternal and maternal genome does not occur in a donor cell nucleus to the same degree (Bourc'his et al., 2001; Dean et al., 2001). Potentially this difference is because gametes are in some way poised for reprogramming, whereas the donor nuclei are not. Data suggest that the greater the amount of differentiation, the less successful is the SCNT process. For example, in amphibians approximately 30% of nuclear transfer performed with blastula cells are successful, compared to only about 2% for differentiated cells (Gurdon and Wilmut, 2011). A similar pattern has been observed
in mammals. Less differentiated cells, such as blastomeres or fetal fibroblasts (Rideout et al., 2000), have greater cloning success than adult fibroblasts or terminally differentiated cells (Hochedlinger and Jaenisch, 2002; Eggen et al., 2004; Li et al., 2004; Inoue et al., 2005). Reprogramming of the oocyte genome is directed by as yet unknown factors in the cytoplasm. The reprogramming capacity of oocytes diminishes shortly after cleavage begins. Beyond the two to four-cell stage, the cytoplasm of blastomeres does not appear capable of reprogramming donor cell nuclei (Wakayama et al., 2000).

Microarray analysis of gene expression immediately following nuclear transfer indicates that an overwhelming majority (95%) of genes are expressed normally in cloned embryos (Humpherys et al., 2002; Smith et al., 2005; Beyhan et al., 2007; Vassena et al., 2007). Based on this evidence, one can reason that reprogramming is largely properly achieved. However, the lack of expression of a few key genes within the minority of non-programmed genes may be sufficient to induce abnormal development (Boiani et al., 2002; Bortvin et al., 2003; Aston et al., 2010).

Cloned mouse embryos derived from cumulus cells can successfully silence cumulus-specific genes, demonstrating the capacity for reprogramming the gene expression patterns of donor cell-specific genes (Tong et al., 2007). This study only examined four genes, and other studies have indicated donor-specific genes maintain expression (Gao et al., 2003; Tong et al., 2006). Taken together, these findings suggest that reprogramming occurs, although reprogramming of specific genes can be incomplete.
In amphibians it has been shown that transferred donor cells retain epigenetic memory. Donor cells derived from the mesoderm, ectoderm or endoderm maintain expression of lineage-specific genes upon reaching the blastocyst stage in over half of cloned blastocysts (Ng and Gurdon, 2008). Such studies highlight that lineage-specific identity of donor nuclei is retained throughout preimplantation development. A comparable experiment has yet to be carried out in mammals, although a study from the mouse suggests that the same may hold true of mammalian cloning. The use of myoblast nuclei as a donor source, followed by embryo culture in myoblast culture medium, triggers the expression of muscle-specific cell markers in cloned embryos (Gao et al., 2003).

The successful development of cloned embryos may hinge on the proper expression of pluripotency factors such as *POU5F1* (Boiani et al., 2002; Bortvin et al., 2003; Byrne et al., 2003; Armstrong et al., 2006; Lee et al., 2006) as preimplantation embryos require *POU5F1* in order to develop (Nichols et al., 1998; Pantazis and Bollenbach, 2012). *POU5F1* is a transcription factor that directs the expression of multiple developmental genes and is essential for early development (Niwa et al., 2000). SCNT embryos fail to upregulate embryo-specific genes including *POU5F1* (Boiani et al., 2002; Bortvin et al., 2003; Kishigami et al., 2006). It has been reported in some mouse studies that the use of ES cells as a donor source have higher cloning efficiency over somatic cells (Wakayama et al., 1999; Rideout et al., 2000; Eggan et al., 2001), which may be due, in part, to expression of *POU5F1* in ES cells (Nichols et al., 1998; Pantazis and Bollenbach, 2012).

Exposure of donor cells to cell extracts designed to induce reprogramming prior to
transfer has had limited success (Landsverk et al., 2002; Hansis et al., 2004), likely because these agents do not discriminate against terminally silenced regions and are not specific to the few genomic regions that require epigenetic activation. Recent work has demonstrated that injecting mouse donor cells into Xenopus eggs leads to demethylation of the mouse POU5F1 gene (Simonsson and Gurdon, 2004). Such an outcome is unexpected, given that the Xenopus zygote does not undergo active demethylation (Stancheva et al., 2002). The results suggest that a conserved, yet to be identified mechanism exists that is capable of genome reprogramming.

Genetic mutations accumulate during aging and during prolonged periods of in vitro culture (DePinho, 2000). This opens the possibility that cloning failure is in part caused by genetic abnormalities. However, the fact that cloning abnormalities are shared among all species, as well the observation that cloned animals are able to give birth to offspring without any mutant phenotype strongly suggests that the deficiencies in cloned organisms are epigenetic in nature. It is unclear however, how much of reprogramming process is mediated by the oocyte environment versus the epigenetic state of the individual donor cell at the time of transfer (Roemer et al., 1997).

In cattle, no difference has been seen between cloning success using donor cells collected from young versus old individuals or low versus high passage fibroblasts (Hill et al., 2000b; Kubota et al., 2000). However a conflicting report from sheep suggests that rates of cloning failure are higher with high passage number fibroblasts (Schnieke et al., 1997; Wilmut et al., 1997; McCreath et al., 2000). However, it is important to note that in these experiments, the overall success rate of any sample was extremely low, making it
difficult to draw statistically significant conclusions.

Although several examples demonstrate that cloned embryos are characterized by reprogramming failures a limited number of cloned offspring appear normal in all regards, suggesting that appropriate reprogramming can occur (Chavatte-Palmer et al., 2002; Cibelli et al., 2002). Additionally, some aspects of embryo development appear to be normal in clones. X-chromosome inactivation occurs following the formation of the zygote. X chromosomes are randomly silenced within ICM cells, but in trophoblast cells, an unknown imprinting mechanism causes preferential inactivation of the paternal X-chromosome. Cloned embryos follow a similar pattern of paternal X-chromosome inactivation in trophoblasts (Eggan et al., 2000), indicating that clones retain this hierarchical silencing process.

**The P53-Mediated Apoptosis Pathway**

The transcription factor P53 plays a central role in cellular health and ensures genomic integrity by initiating programmed cell death within defective and aberrant cells. During P53-mediated apoptosis, the cell shrinks, DNA is fragmented and membrane blebbing occurs. These features allow the degraded cell contents to be phagocytosed, thus preventing inflammation in surrounding tissue that would occur through cell death without apoptosis (Kerr et al., 1972). P53 acts as a sensor to eliminate unneeded cells and prevent overgrowth, as well as eliminating cells under stress, with DNA damage and that grow uncontrollably. Due to its role in enforcing genomic stability, P53 is commonly referred to as the guardian of the genome. By preventing mitotic division of cells with DNA damage or oncogene expression, P53 prevents tumorigenic growth. The
critical role of P53 in preventing growth of aberrant cells is clearly shown by the high rate and early development of tumors in P53 knockout mice (Donehower et al., 1992).

Under normal conditions, cells maintain a constant but low level of P53 within the cytoplasm. Because of its potential to terminate progression through the cell cycle, mechanisms that maintain P53 in an inactive form are strictly regulated. The regulator MDM2 forms a negative feedback loop to prevent P53 activation by consistently targeting P53 for degradation, thereby preventing accumulation of P53 within the cell (Moll and Petrenko, 2003; Kruse and Gu, 2009).

Activation of P53 can be induced by a variety of triggers including DNA damage, hypoxia, nucleotide depletion, reactive oxidative species, and oncogene activation, with the activation method determining the action carried out by P53. Activation of the P53 pathway may occur directly via various input signals, or activation can arise indirectly through the inhibition of MDM2 that leads to the accumulation and subsequent activation of P53. Upon activation, P53 homodimerizes and translocates to the nucleus where it upregulates a number of genes to carry out cell cycle arrest, senescence or apoptosis (Vousden and Lu, 2002).

A number of genes upregulated by P53 have been identified (Riley et al., 2008). Upon activation, P53 can either induce cell cycle arrest or initiate programmed cell death. Under normal conditions, cell cycle progression is induced by cyclin-dependent kinases (CDK), which are upregulated in preparation for progression through different stages of the cell cycle. CDKs form a complex with cyclin which acts as an inducer for the cell cycle. $P21^{WAF1/Cip1}$ ($CDKN1A$) is upregulated by P53 (Brugarolas et al., 1999) and acts
by inhibiting cyclin/CDK to prevent cell cycle progression (Niculescu et al., 1998). Thus, P53 upregulation of $P21^{WAF1/Cip1}$ induces cell cycle arrest and prevents cell division.

Aside from cell cycle arrest, activation of P53-dependent gene expression leads to apoptosis. Alternatively, P53 can directly activate apoptosis in a transcription-independent manner. Either pathway ultimately leads to the release of cytochrome c from the mitochondrial membrane, which acts as a mediator of programmed cell death (Wei et al., 2001). In both transcription-dependent and transcription-independent pathways, the downstream effects of P53 at the mitochondria involve members of the BCL2 family of proteins (Cory and Adams, 2002) that can either activate or inhibit apoptosis by controlling release of cytochrome c from the mitochondria.

Pro-apoptotic members of the BCL2 protein family include BAX, BAK, BID NOXA, and PUMA. These pro-apoptotic proteins can be upregulated by P53 (Oltvai et al., 1993; Miyashita and Reed, 1995) and oligomerize to form a channel on the membrane of mitochondria through which cytochrome c is released (Miyashita and Reed, 1995; Chipuk et al., 2004). Anti-apoptotic members of the BCL2 protein family include BCL-2, BCL-xl, and MCL-1. These proteins antagonize pro-apoptosis inducing proteins. BAX activity is inhibited by BCL2, which heterodimerizes with BAX and inhibits BAX-mediated induction of apoptosis (Yin et al., 1994). The BCL2:BAX ratio controls whether apoptosis is induced or inhibited (Basu and Haldar, 1998). BCL2 is expressed continually and maintains a constant inhibition of BAX. However, upon upregulation of
BAX by P53, the BCL2:BAX ratio is tipped in favor of BAX, allowing BAX to oligomerize and initiate apoptosis.

The P53 pathway may also act in a transcription-independent manner to induce apoptosis (Caelles et al., 1994; Wagner et al., 1994; Yan et al., 1997; Gao and Tsuchida, 1999). Deletion of the DNA-binding domain of P53 maintains its apoptosis-inducing ability under certain conditions (Haupt et al., 1995; Chen et al., 1996; Haupt et al., 1997). When acting as a direct activator of apoptosis, P53 translocates to the mitochondria (Marchenko et al., 2000; Sansome et al., 2001; Mihara et al., 2003) where it initiates apoptosis by either the activation of pro-apoptosis proteins, or the inhibition of anti-apoptosis proteins (Mihara et al., 2003; Tomita et al., 2006). P53 is able to both directly activate BAX and to inhibit BCL2 (Wang et al., 1993; Chiou et al., 1994; Marin et al., 1994; Froesch et al., 1999).

The transcription-independent pathway of P53-directed apoptosis has been linked to apoptosis induction by reactive oxygen species (Han et al., 2008). However, DNA damage response and tumor suppression requires P53 to act in a transcription-dependent manner (Chao et al., 2000; Jimenez et al., 2000; Johnson et al., 2005; Gaidarenko and Xu, 2009). In cells that undergo cell cycle arrest, P53 does not localize to the mitochondria. In this case, cell cycle arrest is a transcription-dependent process (Marchenko et al., 2000; Erster et al., 2004) and involves the upregulation of $P21^{WAF1/Cip1}$.

Via its role in regulating apoptosis, P53 is a vital component of normal development and maintenance of tissue and organs. For example, P53-null mice have a
high incidence of birth defects, such as polydactylism, problems in bone development, and abnormalities in neural tube formation (Armstrong et al., 1995).

P53-mediated apoptosis also plays a role during preimplantation development (Hardy et al., 2001; Gjorret et al., 2003). P53 prevents the integration of blastomeres with genetic abnormalities (Fabian et al., 2005). Conversely, excessive accumulation of P53 within the developing embryo is a direct impediment to embryo viability and implantation (Ganeshan et al., 2010). In mouse preimplantation embryos, decreased apoptosis levels result in significantly higher rates of fetal development following embryo transfer (Li et al., 2007).

Recently, P53 was revealed to play a major role in the efficiency of reprogramming iPSCs, as the elimination of P53 dramatically improved reprogramming efficiency (Zhao et al., 2008). P53-null cell lines have dramatically higher success rates of reprogramming (Hong et al., 2009; Kawamura et al., 2009). Additionally, cell lines that have previously failed to reprogram have been able to successfully do so following p53 inhibition (Utikal et al., 2009).

The reprogramming pathway common in iPSC formation induces apoptosis, senescence, and cell cycle arrest. Reprogramming causes DNA damage, which can activate the P53 apoptosis pathway (Marion et al., 2009). Additionally, the reprogramming factors used to induce somatic cell potency also have oncogenic effects that likely induce apoptosis. The six factors regularly used in reprogramming experiments, C-MYC, LIN28, KLF4, POU5F1, NANOG, and SOX2 (Takahashi et al., 2007; Yu et al., 2007; Silva et al., 2009), are also involved in the induction of apoptosis.
C-MYC is an established oncogene that induces apoptosis when overexpressed (Pelengaris et al., 2002; Rowland and Peeper, 2006). P53-null cell lines can be induced to become stem cells by the addition of the reprogramming factors POU5F1 and SOX2 alone, suggesting that one function of C-MYC in reprogramming may be to select for cells with reduced P53 functionality (Eischen et al., 1999; Kawamura et al., 2009). LIN28 is a translational enhancer of various oncogenes, including C-MYC (West et al., 2009), and its presence likely enhances C-MYC expression and increases apoptosis. KLF4 suppresses P53 (Rowland and Peeper, 2006), while simultaneously inducing the expression of $P21^{WAF1/Cip1}$ to inhibit cell cycle progression (Zhang et al., 2000; Chen et al., 2003; Rowland et al., 2005; Rowland and Peeper, 2006). NANOG, POU5F1, and SOX2 are required for potency and are overexpressed in some types of cancers (Almstrup et al., 2004; Ezeh et al., 2005; Hart et al., 2005; Hochedlinger et al., 2005). P53 is activated by these factors either collectively (Hong et al., 2009; Kawamura et al., 2009; Marion et al., 2009), or individually (Kawamura et al., 2009). Additionally, SOX2 and POU5F1 act to trigger cell cycle arrest by upregulating $P21^{WAF1/Cip1}$ via P53 induction (Kawamura et al., 2009).

As SCNT requires reprogramming of the somatic donor cell, the activation of P53-mediated apoptosis may be responsible for increased cell death following somatic cell nuclear transfer. However, no studies have established the expression of reprogramming associated genes or the upregulation of P53 targets following SCNT. Apoptosis has been reported to occur with higher frequency in embryos produced by SCNT than embryos produced by IVF (Fahrudin et al., 2002; Hao et al., 2003; Jang et al., 2005).
2004; Gjorret et al., 2005; Im et al., 2006; Kumar et al., 2007), and elevated apoptosis continues throughout development (Hao et al., 2003). However, it is unclear if increased apoptosis is the result of sub-optimal culture conditions, the stress associated with the cloning procedure, or possibly the byproduct of donor cell reprogramming. The cloning procedure may induce higher levels of apoptosis. For example, porcine clones activated by electroporation alone have reduced apoptosis relative to clones activated by electroporation with chemical activation (Im et al., 2006).

In addition to increased levels of apoptosis, cloned embryos have a reduced number of blastocyst cells that likely is due to increased apoptosis (Koo et al., 2000; Fahrudin et al., 2002; Gjorret et al., 2005; Terashita et al., 2011). A reduced blastomere number likely results in reduced embryo viability and implantation potential (van Soom et al., 1997; Wuu et al., 1999). Both apoptosis and total cell count reflect important developmental parameters that influence the developmental potential of preimplantation embryos (Brison and Schultz, 1997; Brison and Schultz, 1998; Koo et al., 2000; Knijn et al., 2003). Should the reprogramming process in SCNT embryos activate P53-mediated apoptosis, this would suggest that the high rates of embryo failure might, in part, be the result of donor cell reprogramming.

**Summary**

A great deal of effort by many research groups has been applied to improving the efficiency of SCNT. A large body of evidence supports the hypothesis that clones suffer from a failure to reset the epigenetic program of the somatic donor cell. There is also evidence that preimplantation SCNT embryos experience elevated levels of apoptosis,
which likely contributes to failed embryo development and implantation. A separate line of inquiry has demonstrated that somatic cell reprogramming activates the P53 apoptosis pathway and induces programmed cell death in cells that are aberrantly reprogrammed within the somatic cell genome. Should the same pattern of reprogramming-induced apoptosis occur in cloned embryos, then SCNT embryo failure may be the result of successful reprogramming, rather than a reprogramming failure.

Research Goals and Potential Applications

The first objective of my dissertation was to characterize the patterns of DNA methylation in SCNT embryos, in order to identify the epigenetic reprogramming status of several important developmental genes following SCNT, and to understanding the regulation of DNA methylation. The second objective was to characterize p53-mediated apoptosis following SCNT.

For the first objective, I was able to demonstrate that reprogramming is incomplete following SCNT, and that methylation patterns at some CpG sites in key genes of SCNT embryos are more similar those found in the donor cell than those seen in IVF embryos. In a separate study related to DNA methylation, I characterized the methylation status of a family of LIN28 pseudogenes. These studies hold the potential to better understand how the addition and maintenance of methylation patterns are regulated.

For the second objective, I confirmed the activation by P53-mediated apoptosis within SCNT preimplantation embryos, and identified several P53-activated genes that are upregulated in clones. Importantly, my work demonstrated that the reprogramming
factors C-MYC, SOX2, KLF4, and LIN28 are upregulated in clones. My findings suggest that SCNT embryos have elevated expression of reprogramming factors. The expression of these factors likely activates P53, leading to increased apoptosis and cell cycle arrest in SCNT embryos. These findings identify several genes that contribute to blastomere depletion and failed development.

SCNT holds immense potential in the production of transgenic animals, the generation of stem cells, and the production of species with superior traits. However, all potential benefits are constrained by the low efficiency of the technique. My research strengthens our understanding of the nature of embryo failure, and reveals potential interventions to inhibit apoptosis to allow SCNT with higher developmental rates. Additionally, my findings in the area of DNA methylation contribute to greater understanding of the reprogramming process and have application in several areas of biology, including stem cell biology, reprogramming, epigenetics, and cancer.

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CHAPTER 2
DNA METHYLATION REPROGRAMMING OF THE POU5F1, NANOG, SOX2 AND KLF4 GENES FOLLOWING SOMATIC CELL NUCLEAR TRANSFER

Abstract
Aberrant methylation patterns have been implicated as a contributing factor in the poor development of embryos following somatic cell nuclear transfer (SCNT). Embryos produced via SCNT that are unable to epigenetically reprogram somatic cell-specific methylation patterns are at a developmental disadvantage, as the donor cell methylation signature retained within cloned embryos reinforces inappropriate gene silencing. Using bisulfite sequencing, we compared multiple methylation sites in the POU5F1 (OCT4), NANOG, SOX2, KLF4, C-MYC, and LIN28 genes within bovine IVF and SCNT blastocyst embryos and donor cells. Prior to implantation, the methylation patterns of NANOG and SOX2 in SCNT embryos are more similar to patterns observed in somatic cells than patterns seen in IVF blastocysts. We also observed that methylation of POU5F1 is lower in SCNT blastocysts, suggesting that reestablishment of methylation of this gene prior to implantation of SCNT embryos is delayed. Our results demonstrate that methylation patterns vary among developmental genes in SCNT embryos, with reprogramming at some CpG sites and incomplete reprogramming at others.

Introduction
Since the cloning of the first animal from somatic cells, the technique of somatic cell nuclear transfer (SCNT) has had limited success in embryo development and
production of viable offspring (Wilmut et al., 1997). Despite intense research, the benefits of cloned offspring remain limited due to the failed development of embryos and fetuses generated via SCNT (Wells, 2005). Following SCNT, many cloned neonates die shortly after birth and often exhibit phenotypic abnormalities (Hill et al., 2000a; Farin et al., 2004; Li et al., 2005). SCNT-derived organisms face challenges at each stage of development. The low success rate of the SCNT procedure appears to be due in large part to incomplete embryonic reprogramming of the somatic genome (Wilmut et al., 1998; Colman, 1999; Kikyo and Wolffe, 2000; Solter, 2000; Dean et al., 2001; Humpherys et al., 2001; Reik and Walter, 2001; Niemann et al., 2008).

Several phenotypic abnormalities have been observed in clones, including placental deficiency, fetal loss, respiratory problems, and cardiovascular failure (Hill et al., 1999; Hill et al., 2000b; De Sousa et al., 2001; Chavatte-Palmer et al., 2002; Yang et al., 2007). Neonates are predisposed to infection and weakness (Zakhartchenko et al., 2001), as well as a condition referred to as large offspring syndrome, which is characterized by abnormally large body and organ size (Young et al., 1998; Farin et al., 2004). Large offspring syndrome shares many similarities with human epigenetic disorders, such as Beckwith-Wiedman syndrome (DeBaun et al., 2003; Everts et al., 2008). In addition, altered levels of gene expression have been observed in SCNT embryos (Sebastiano et al., 2005; Aston et al., 2010). These abnormalities are likely due to the failure of the SCNT embryo to appropriately reprogram the epigenome of the donor cell nucleus to an embryonic state.
DNA methylation in mammals involves the covalent addition of a methyl group to the cytosine within cytosine-guanine (CpG) dinucleotides. DNA methylation is responsible for the inactivation of transposons within the genome (Yoder et al., 1997), X-chromosome inactivation (Mohandas et al., 1981; Csankovszki et al., 2001), establishment and silencing of imprinted genes (Bartolomei et al., 1993; Li et al., 1993; Stoger et al., 1993), and maintenance of chromosome stability (Chen et al., 1998). Additionally, DNA methylation plays a role in the silencing of gene transcription (Jaenisch and Bird, 2003; Shiota, 2004) through one of two mechanisms: 1) the presence of methyl groups in DNA directly blocks the binding of transcription factors (Watt and Molloy, 1988; Hendrich and Bird, 1998; Prokhortchouk et al., 2001) and 2) methylation of DNA targets the region for chromatin silencing that prevents gene transcription (Jones et al., 1998; Wade et al., 1999; Zhang et al., 1999; Sarraf and Stancheva, 2004).

Although any CpG site within the mammalian genome can incur methylation (Bird, 2002), CpG dinucleotides often cluster within CpG dense regions known as CpG islands (Illingworth and Bird, 2009). These islands are commonly found at the 5’-end of genes, have a high density of CpG sites (about one site per 10 bp), and are associated with transcriptional start sites (Antequera and Bird, 1993).

Abnormal levels of DNA methylation present a significant impediment to animal cloning. Cloned bovine embryos have higher levels of global methylation than in vivo or in vitro fertilized (IVF) embryos (Kikyo and Wolffe, 2000; Kang et al., 2001). Additionally, specific genes have been shown to undergo gradual loss of methylation in cloned embryos throughout embryo development (Yamazaki et al., 2006), indicating that
methylation patterns of somatic cells are not correctly reprogrammed via the same mechanism undergone by *in vivo* and IVF embryos.

The discovery of transcription factors capable of inducing pluripotency in differentiated cells may provide insights into the challenges of SCNT development (Takahashi et al., 2007; Yu et al., 2007). Pluripotency-inducing transcription factors include *POU5F1* (*OCT-4*) (Pesce and Scholer, 2001), *NANOG* (Chambers et al., 2003; Mitsui et al., 2003), *SOX2* (Avilion et al., 2003), *KLF4* (Evans and Liu, 2008), *C-MYC* (Adhikary and Eilers, 2005), and *LIN28* (Darr and Benvenisty, 2009). When expressed ectopically in various combinations, these pluripotency genes are able to fully reprogram somatic cells (Takahashi et al., 2007; Yu et al., 2007). Additionally, *POU5F1*, *SOX2*, *KLF4*, *C-MYC*, and *NANOG* expression is essential for the creation of induced pluripotent stem cells in cattle (Sumer et al., 2011). Either the absence or irregular expression of these factors likely impedes successful development following SCNT.

To determine whether or not DNA methylation patterns in pre-implantation stage SCNT embryos are appropriate for embryonic development, we characterized DNA methylation patterns of *POU5F1*, *NANOG*, *SOX2*, *KLF4*, *C-MYC*, and *LIN28* genes in bovine SCNT and IVF blastocysts and compared them to patterns observed in the donor cell line. We determined that the methylation patterns of SCNT embryos are more similar to those of the donor cell for the *NANOG* and *SOX2* genes. Additionally we determined that the methylation patterns of CpG sites in *POU5F1* were abnormally regulated in SCNT embryos. The aberrant epigenetic patterns may lead to irregularities in gene expression patterns and restrict reprogramming in SCNT embryos.
Materials and Methods

Donor Cell Culture

Bovine fibroblasts were collected from skin samples and cultured in DMEM F12 (Thermo Scientific HyClone Laboratories, Logan, UT) supplemented with 15% fetal bovine serum (FBS) (Thermo Scientific HyClone Laboratories), 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were seeded in T25 culture flasks and cultured at 37°C with 5% CO₂. Prior to SCNT cultured cells were treated with 0.25% trypsin and resuspended in phosphate buffered saline (PBS).

Oocyte Maturation

Cumulus oocyte complexes were aspirated from 3 to 8 mm follicles from ovaries collected at a local abattoir (E.A. Miller, Hyrum, UT). Cumulus oocyte complexes were cultured 18 to 22 hr in TCM 199 maturation medium (Thermo Scientific HyClone Laboratories) containing 10% FBS, 0.05 mg/ml follicle stimulating hormone (FSH), 5 mg/ml luteinizing hormone (LH), 100 U/ml penicillin, and 100 mg/ml streptomycin.

In Vitro Fertilization

Following 18 to 22 hr of oocyte maturation, cryopreserved bovine semen (Hoffman AI, Logan, UT) was thawed and live sperm were collected via centrifugation with a 45%/90% percoll gradient. Live sperm were suspended in Tyrode’s albumin lactate pyruvate (TALP) to be used for oocyte fertilization. Twenty-four hours following fertilization, cumulus oocyte complexes were vortexed in PBS with 0.32 mM sodium pyruvate, 5.55 mM glucose, 3 mg/ml BSA, and 10 mg/ml hyaluronidase to remove...
cumulus cells from the oocyte. Oocytes were subsequently washed with PBS and cultured in CR2 medium atop a layer of cumulus cells.

**SCNT Production**

Cumulus cells were removed following maturation as described above. Metaphase II oocytes with an extruded first polar body were selected as recipient cytoplasts. Following enucleation, removal of the metaphase plate and polar body, a single donor cell was placed in the perivitelline space. Oocyte and donor cell fusion was carried out with two electric DC pulses of 2.2 kV/cm for 25 µsec applied to embryos placed in a fusion chamber suspended in mannitol fusion medium (Wells et al., 1999). Following fusion, embryos were placed in CR2 medium for 1 to 3 hr prior to activation. Embryo activation was performed 23 to 25 hr following maturation of oocytes by treatment with 5 mM ionomycin for 5 min and 10 mg/ml cyclohexamide for 5 hr. Activated embryos were then placed in co-culture dishes with CR2 medium atop a layer of cumulus cells.

**Bisulfite Conversion**

A pool of 25 SCNT or IVF embryos was collected over the course of 3 IVF or cloning sessions. Embryos were collected on day eight of development, and embryos of similar size and quality were selected. Following collection embryos were snap frozen with liquid nitrogen and stored at -80°C until direct bisulfite conversion. Approximately 1 million donor cells were collected and DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD) and 1 µg of DNA was used for bisulfite
conversion. Embryos were used directly for bisulfite conversion. Bisulfite conversion was carried out using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA) according to the manufacturer’s protocol. Converted DNA was immediately used in PCR reactions.

*PCR and 454 Sequencing*

Following bisulfite conversion, converted embryo pools or donor cell DNA were used as template in 25 µl PCR reactions using 1 to 3 µl bisulfite converted genomic DNA with 0.6 µM primers in GoTaq Master Mix (Promega, Madison, WI) reactions. The same cycling parameters were used for all primer pairs: 94°C for 1 min, annealing temperature ranging from 54 to 59°C for 20 sec, and 72°C for 30 sec for 30 cycles. A second PCR was performed using identical primers including the 454 adapter and key sequence (adapter A CCATCTCATCCCTGGCTGTCTCCGACTCAG on the forward primer and adapter B CCTATCCCTGCTGTGCCTTGAGTCGCTAGCTTCAG on the reverse primer). This second round of PCR used 1 to 3 µl of the PCR product from the first round and 0.3 μM primer in GoTaq Master Mix (Promega) and the following cycling parameters: 94°C for 30 sec, annealing temperature ranging from 55 to 60°C for 20 sec, and 72°C for 30 sec for 15 cycles. After purification with AMPure beads (Beckman Coulter, Brea, CA) and quantification with PicoGreen (Life Technologies, Carlsbad, CA), PCR products were pooled and finally sequenced on the 454 GS FLX Titanium DNA sequencer (Roche, Indianapolis, IN) according to the manufacturer’s protocols. Briefly, amplicon libraries were subjected to emulsion PCR to produce DNA-coated
beads, loaded onto a 70X75 PicoTiterPlate, and sequenced using the FLX Titanium DNA sequencing Kit.

Sequence Analysis

Sequencing data was analyzed for frequency of methylation using BISMA analysis software (Rohde et al., 2010). Data are expressed as the percentage of methylated CpG sites by normalizing methylation frequency data to the number of sequence reads obtained.

Statistical Analysis

To determine whether methylation levels were significantly different among donor cells, IVF, and SCNT embryos, we performed Fisher’s exact tests on a per CpG site basis using the number of methylated transcripts and the number of unmethylated transcripts in a categorical analyses; thus, these tests are influenced by the total number of transcripts obtained for each amplicon. The resulting raw $P$ values were then Bonferroni-adjusted using proc multtest (SAS 9.3, Cary, NC) to account for multiple testing.

Results

DNA methylation patterns were examined using bisulfite sequencing of 336 CpG sites at single base pair resolution across six genes. DNA methylation within the donor cell line was observed in the $POU5F1$, $NANOG$, and $SOX2$ genes, whereas limited methylation was observed within the gene body of $KLF4$. The $LIN28$ and $C-MYC$ genes had no methylation within any of the regions examined and were excluded from further
analysis. Once the donor cell methylation patterns were examined, patterns of \textit{POU5F1}, \textit{NANOG}, \textit{SOX2}, and \textit{KLF4} within IVF and SCNT blastocyst stage embryos were examined. This analysis revealed aberrant methylation of DNA within SCNT embryos.

\textit{Methylation of POU5F1}

Analysis of the \textit{POU5F1} CpG island revealed an island beginning immediately upstream from the translation start site that spans the entirety of the first exon (Fig. 2-1). The methylation status of the \textit{POU5F1} CpG island is summarized in Figure 2-2. Overall, 54\% of the CpG sites were methylated within this region. Interestingly the methylation levels were not uniform across the length of the CpG island, as relatively low methylation was detected at the 5’ end and comparatively high methylation at the 3’ end of the examined sequence. Overall methylation in IVF embryos was low, with 35\% of all CpG sites methylated. Methylation in bovine SCNT embryos was substantially lower than both donor cells and IVF blastocysts, with 18\% of all CpG sites methylated (Fig. 2-2).

A statistical comparison of these methylation profiles revealed specific CpG sites that are differentially methylated. CpG site 4 is among the most frequently methylated sites in the donor cell line (Fig. 2-2). Seventy-one percent of donor cell sequence reads are methylated, compared to 49\% in IVF embryos. By contrast, none of the SCNT sequence reads were methylated at this site. Similar observations were observed at sites 26 and 40, all of which were differentially methylated in SCNT embryos and donor cells (Fig. 2-2). These CpG sites appear to have undergone successful reprogramming within the SCNT genome, although based on methylation patterns observed within IVF samples, the reduction of methylation levels appears to be inappropriate for the blastocyst stage.
In SCNT embryos, methylation at the 3’ end of the CpG island is more prevalent compared to the 5’ end, although methylation of this 3’ region remains lower than levels detected in IVF embryos. Of note, methylation at CpG sites 140, 164, and 263 is significantly different lower in SCNT embryos compared to IVF embryos or to donor cells (Fig. 2-2). At a number of other CpG sites in this 3’ region, methylation levels are significantly different among all sample types (e.g., sites 232, 250, 284, 320, 355).

When considering the entire CpG island, average methylation levels are highest in donor cells (54%), lower in IVF embryos (35%), and lowest in SCNT embryos (18%). However, three closely aligned CpG sites break from this apparent pattern. Specifically CpG dinucleotides at sites 56, 64, and 79 are more frequently methylated in SCNT embryos than IVF, yet methylation at these sites is lower in both embryo types compared to donor cells (Fig. 2-2).

*Methylation of NANOG*

*NANOG* contains a small CpG island within the first intron that begins 270 bp downstream from the transcription start site (Fig. 2-1). The methylation status for this region is summarized in Figure 2-3. Within this region, some SCNT CpG sites more closely resemble the methylation pattern of the donor cell, while others are more similar to IVF. The overall average DNA methylation for this region is higher in the SCNT samples than donor cell as well as IVF. Within the donor cell samples, 25% of CpG sites are methylated; in IVF blastocysts, 21% of CpG dinucleotides are methylated; and within SCNT blastocysts, 31% of CpG sites are methylated (Fig. 2-3). Results of the statistical analyses of methylation frequency were not significant for any particular CpG site, likely
a consequence of the low number of sequence reads obtained. Even so, it is interesting to note that for seven CpG loci, methylation is completely absent in IVF embryos, while several of these sites (e.g., 303, 308, 487) are moderately methylated in SCNT embryos and donor cells. Only one site was not methylated in SCNT embryos, while every CpG locus examined was methylated to some extent in donor cells.

Another interesting feature of the examined region in *NANOG* was an apparent spike in the percentage of methylated cytosines at CpG site 523. A methylation spike is defined here as a sharp elevation in the methylation of a CpG dinucleotide that is closely associated with other CpG loci that retain low levels of methylation. Within the *NANOG* CpG island site 523 was methylated at a percentage of 83% in SCNT embryos, 70% in donor cells, and 55% in IVF embryos (Fig. 2-3).

*Methylation of SOX2*

*SOX2* is a single exonic gene and contains a 3000 bp CpG dense region that begins 1500 bp upstream from the transcriptional start site and continues throughout the gene. Three CpG islands were identified within this region, and several CpG sites were examined for methylation (Fig. 2-1). Our analysis identified two regions that were positive for methylation within donor cells, and both regions were subsequently examined for methylation patterns within IVF and SCNT embryos. Results are summarized in Figure 2-4.

The first region is upstream from the translational start site and ranges from -743 bp to -480 bp. This region is hypomethylated at all CpG sites with the exception of a cluster of four CpG dinucleotides from -598 bp to -585 bp. For all three sample types, a
similar pattern of methylation was observed in the sequence of these four CpG sites contained within a 14 bp segment of DNA, where sites -598, -589, and -585 are methylated with moderate to high frequency (38 to 100%), while site -593 is not methylated for any sequence read examined in any of the tissue types (Fig. 2-4). Interestingly, methylation of these sites seems to be maintained in a specific pattern across tissue types, where sites -598, -589, and -585 are methylated with 100% frequency in SCNT embryos, 63 to 70% frequency in donor cells, and only 38 to 50% frequency in IVF embryos. Also within this region, CpG site -701 is appears more frequently methylated in SCNT embryos and donor cells compared to no methylation in IVF embryos.

A second region within SOX2 ranging from 600 bp to 1400 bp downstream from the transcriptional start site was methylated to varying degrees in donor cells, IVF embryos and SCNT embryos. A second cluster of highly methylated sites exists within this region, and methylation for this sequence of CpG sites (562-669) is consistent for IVF and SCNT embryos and higher than that of donor cells for sites 652 and 669. Few statistical differences were detected for CpG sites in the SOX2 gene due to the relatively low number of reads obtained.

Methylation of KLF4

Two large CpG islands are associated with the KLF4 gene ranging from 1800 bp upstream to 2200 bp downstream from the transcriptional start site in the third intron (Fig. 2-1). In all sample types, methylation of the regions from -1218 to -361 and +1250 to +1900 was quite low, averaging from 1 to 9% (Fig. 2-5). The region upstream from
the transcriptional start site was not methylated at any CpG site in any of the tissue types examined (data not shown). The region downstream of the transcriptional start site, from 1553 to 1884, was methylated to a limited extent in donor cells, but only minimally methylated at a few loci in IVF or SCNT embryos. The donor cell likewise has minimal methylation throughout the region (Fig. 2-5). Interestingly, significant differences in frequency of methylation were observed for many CpG sites among the tissue types, even though the differences were not large in magnitude. Most notable were differences in donor cells and IVF embryos from sites 1731 to 1806, as well as differences between SCNT embryos or donor cells compared to IVF embryos in the region from 1281 to 1495 (Fig. 2-5).

**Discussion**

The aim of this project was to determine whether the DNA methylation patterns of *POU5F1*, *NANOG*, *SOX2*, *KLF4*, *LIN28*, and *C-MYC* were appropriately reprogrammed in SCNT embryos prior to implantation. We accomplished this objective by measuring DNA methylation of the CpG islands associated with each gene in SCNT and IVF blastocysts. We also measured the methylation status of the donor cell line to determine whether methylation patterns of SCNT embryos more closely resembled those of the donor cell line or IVF embryos. The results of this study suggest that DNA methylation patterns of *POU5F1*, *NANOG*, and *SOX2* in SCNT embryos are abnormally established at several CpG sites in pre-implantation SCNT embryos. Across the broad CpG islands examined, levels of methylation of the *NANOG* and *SOX2* genes were elevated and more closely matched the methylation patterns observed in donor cells. On
the other hand, *POU5F1* is relatively hypomethylated compared to donor cells and IVF embryos; this observation suggests that while reprogramming of *POU5F1* occurs in SCNT embryos, the timing may be delayed compared to IVF embryos.

Several studies have examined the methylation status of CpG sites proximal to the transcriptional start sites for *POU5F1, NANOG*, and *SOX2* genes and observed abnormal methylation patterns in SCNT embryos (Yamazaki et al., 2006). Aberrant epigenetic patterns likely contribute to cloning failure during preimplantation development and early gestation (Bourc'his et al., 2001; Dean et al., 2001; Kang et al., 2001; Ohgane et al., 2001; Kang et al., 2002). In this study, we examined previously uncharacterized CpG islands associated with the *POU5F1, NANOG*, and *SOX2* genes, as well as three other genes not previously studied in bovine embryos, *KLF4, LIN28*, and *C-MYC*. These later three genes were selected because they are likely to be involved in donor cell reprogramming (Takahashi et al., 2007; Yu et al., 2007). CpG islands may or may not directly regulate expression, but can be methylated and are useful in determining the reprogramming status of an associated gene.

DNA methylation is tightly regulated during embryonic development in order to enable proper gene expression and to reestablish appropriate epigenetic patterns during tissue differentiation. Following fertilization, the maternal genome is passively demethylated throughout early embryonic stages (Monk et al., 1987; Howlett and Reik, 1991; Rougier et al., 1998), while the paternal genome is actively demethylated immediately following fertilization (Mayer et al., 2000; Oswald et al., 2000). SCNT embryos are susceptible to disruption of methylation patterns (Kikyo and Wolffé, 2000;
Kang et al., 2001; Bonk et al., 2008). The demethylation that occurs in normal developing embryos is incomplete and abnormally timed in clones (Armstrong et al., 2006).

As expected, CpG sites associated with the \textit{POU5F1} gene are more frequently methylated in donor cells than in IVF embryos. This difference suggests that reprogramming of the epigenome for \textit{POU5F1} is underway in IVF embryos, likely a consequence of ongoing cellular differentiation, yet the process is not quite complete. Alternatively, the overall lower level of methylation for CpG sites associated with \textit{POU5F1} in SCNT embryos suggests that the timing of reprogramming in clones may be delayed compared to IVF blastocysts. Moreover, the disrupted methylation pattern may contribute to the abnormal expression of \textit{POU5F1} observed in clones (Boiani et al., 2002; Bortvin et al., 2003; Aston et al., 2010). Because \textit{POU5F1} is critical for early development (Nichols et al., 1998; Niwa et al., 2000), abnormal expression of this gene imposes an impediment to normal development. Yamazaki et al. (2006) showed that reprogramming of \textit{POU5F1} occurs gradually throughout development, and our results confirm that reprogramming of methylation patterns does indeed occur. The relatively low level of overall methylation for SCNT embryos may be the result of gradual demethylation of the \textit{POU5F1} gene during early embryo development. Alternatively, the low abundance of methylated CpG sites in SCNT embryos may be a consequence of fewer trophectoderm cells present in cloned embryos (Koo et al., 2002). Due to ongoing active cellular differentiation, one would expect higher levels of methylation for pluripotency factors in trophectoderm cells (Hattori et al., 2004). A loss of
trophectoderm cells in cloned embryos, which are often smaller in size compared to IVF embryos, could conceivably influence the proportion of methylated CpG sites acquired from whole embryo samples. Interestingly, one cluster of CpG sites within *POU5F1* was identified for which methylation frequency was substantially higher in SCNT embryos compared to IVF. However, the role of these specific CpG sites in regulating gene expression is, as yet, unclear nor is it known whether these loci are resistant to reprogramming or sensitive to the reestablishment of methylation within the blastocyst stage. Overall, the methylation patterns observed for *POU5F1* are atypical of those observed in IVF and highlight the aberrant nature of the SCNT reprogramming among specific genes.

*NANOG* is involved in the maintenance of pluripotency during embryo development, prevents differentiation (Chambers et al., 2003), and is essential to early development. Aberrant *NANOG* methylation may hinder expression in the early embryo and perturb SCNT embryo development. However, in this study, the overall patterns of methylation across donor cells, IVF and SCNT embryos were broadly similar and relatively moderately methylated (with a few exceptions for IVF embryos where no methylation was detected). Interestingly, *NANOG* contains a CpG site at location 523 characterized by a sudden increase in methylation, whereas neighboring sites were only moderately methylated. That higher levels of methylation were consistently maintained for this site in all sample types suggest that CpG site 523 may be functionally important, yet additional experiments would be necessary to establish a specific role for this particular locus in regulating *NANOG* expression.
SOX2 maintains potency and directs cell lineage fate in combination with POU5F1 (Avilion et al., 2003). SOX2 contains a CpG island upstream from the translational start site that is largely hypomethylated in donor cells, IVF and SCNT embryos. An exception to this regional hypomethylation is a 14 bp sequence approximately 600 bp upstream from the translational start site that contains four CpG sites. Three of these loci are moderately methylated in donor cells and IVF embryos and highly methylated in SCNT embryos, whereas the fourth site is void of methylation in all tissue types. From this data set, it is not possible to know whether or not the elevated methylation at the three CpG sites in this region is a consequence of incomplete reprogramming or premature establishment of hypermethylation for this pluripotency gene in cloned embryos. The apparent tight regulation of methylation within this small region of DNA, as well as the absence of methylation on surrounding CpG dinucleotides suggests that this sequence of CpG sites may play a key regulatory role for SOX2 expression. An analysis of this region of the SOX2 gene promoter using PROMO (Messeguer et al., 2002) revealed that eight transcription factors have affinity for this sequence: SP1, Mad, fa-fg, BTEB3, ADR1, GAGA Factor, P53, and Pax-6. Interestingly, Pax-6 is known to regulate SOX2 expression (Wen et al., 2008). Future studies should focus on the impact of high methylation at these three CpG sites in the promoter for SOX2 on recruitment of critical transcription factors, such as Pax-6, and subsequent expression of the SOX2 gene.

Within the donor cell line, we observed a high degree of variation of methylation among individual cells. There was an average of 125 reads among donor samples, which
generated a large enough data set to see the methylation pattern of several hundred cells gathered from a pool of potential donor cells. This allowed us to look at the possible effect of random donor cell selection. What we observe is that among the genes examined, a small percentage of donor cells lack methylation. In the case of the upstream region of \textit{SOX2}, 26\% of donor cells contain no DNA methylation at any site. As methylation at these locations may carry over from the donor cells within \textit{SOX2}, the lack on methylation on in 26\% of donor cells suggests that some donor cells transferred during SCNT are unmethylated at these CpG sites and the clone would inherit an unmethylated pattern. This may account for why some clones are better prepared for early development.

Overall, we examined 336 CpG sites across six genes involved in reprogramming and early development SCNT and IVF embryos. We found that SCNT embryos harbor some aberrations in patterns of DNA methylation, although these abnormalities were often localized to small regions or specific CpG sites. The most profound evidence for inappropriate genome reprogramming was obtained for the \textit{POU5F1} gene, which was overall hypomethylated compared to IVF embryos. Future work should aim to determine the mechanism(s) responsible for appropriate reprogramming of \textit{NANOG} and \textit{SOX2}, and to determine whether or not this mechanism is defective or inefficient for reprogramming the \textit{POU5F1} gene. Furthermore, future work should establish the consequences of aberrant methylation following implantation and determine the regulatory nature of the three CpG sites identified within the promoter region of \textit{SOX2}. 
References


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Figure 2-1. Map of CpG islands and regions examined for methylation. Horizontal line shows the first 2000 bp of the gene as well as 2000 bp upstream from the translational start site. White boxes show exons. Vertical lines represent CpG sites and grey boxes represent CpG islands. Arrows indicate primers of the regions examined for methylation analysis.
Figure 2-2. Heat map depicting methylation profile of POU5F1 in donor cells and blastocyst stage SCNT and IVF embryos. The reads column indicates the total number of reads associated with each amplicon. The average percent methylation across all CpG sites examined is also shown. Position number specifies the bp location of the CpG site in relation to the translational start site. Each heat map cell indicates the percentage of CpG sites positive for methylation. Red corresponds to high methylation and blue to low methylation. The significant difference table below each heat map panel provides the results of Fisher’s exact tests for each CpG site for the indicated pair-wise comparison with Bonferroni correction for multiple testing (SAS). A stared box indicates a statistically significance difference in methylation ($P <0.05$).
**Figure 2-3. Heat map depicting methylation profile of** **NANOG** **in donor cells and blastocyst stage SCNT and IVF embryos.** The reads column indicates the total number of reads associated with each amplicon. The average percent methylation across all CpG sites examined is also shown. Position number specifies the bp location of the CpG site in relation to the translational start site. Each heat map cell indicates the percentage of CpG sites positive for methylation. Red corresponds to high methylation and blue to low methylation. The significant difference table below each heat map panel provides the results of Fisher’s exact tests for each CpG site for the indicated pair-wise comparison with Bonferroni correction for multiple testing (SAS). A stared box indicates a statistically significance difference in methylation ($P < 0.05$).
**Figure 2-4.** Heat map depicting methylation profile of **SOX2** in donor cells and blastocyst stage SCNT and IVF embryos. The reads column indicates the total number of reads associated with each amplicon. The average percent methylation across all CpG sites examined is also shown. Position number specifies the bp location of the CpG site in relation to the translational start site. Each heat map cell indicates the percentage of CpG sites positive for methylation. Red corresponds to high methylation and blue to low methylation. The significant difference table below each heat map panel provides the results of Fisher’s exact tests for each CpG site for the indicated pair-wise comparison with Bonferroni correction for multiple testing (SAS). A stared box indicates a statistically significance difference in methylation ($P < 0.05$).
### Figure 2-5. Heat map depicting methylation profile of KLF4 in donor cells and blastocyst stage SCNT and IVF embryos.

The reads column indicates the total number of reads associated with each amplicon. The average percent methylation across all CpG sites examined is also shown. Position number specifies the bp location of the CpG site in relation to the translational start site. Each heat map cell indicates the percentage of CpG sites positive for methylation. Red corresponds to high methylation and blue to low methylation. The significant difference table below each heat map panel provides the results of Fisher’s exact tests for each CpG site for the indicated pair-wise comparison with Bonferroni correction for multiple testing (SAS). A stared box indicates a statistically significance difference in methylation ($P < 0.05$).

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CHAPTER 3

DNA METHYLATION OF THE LIN28 PSEUDOGENE FAMILY

Abstract

DNA methylation is a covalent attachment on DNA that directs epigenetic silencing of selected regions of DNA. Methylation is widespread throughout the genome and contributes to various forms of genomic regulation. Pseudogenes are decayed copies of duplicated genes that spread throughout the genome by retrotransposition. Pseudogenes are transcriptionally silenced by DNA methylation, but little is known about the mode of epigenetic regulation of pseudogenes. Using bisulfite next generation sequencing we examined the methylation status of four pseudogenes of the LIN28 family along with the parent gene to determine the methylation status of dispersed pseudogenes sharing a common origin. Our aim was to determine whether pseudogenes derived from LIN28 maintain the same pattern of methylation as the LIN28 parent gene or acquire a methylation pattern that reflects the region of insertion. Results of this study indicate that pseudogenes are methylated in patterns specific to the local genomic environment and do not recapitulate the methylation signature of the parent gene.

Introduction

DNA methylation is utilized to control diverse aspects of genome regulation and transcriptional activity. Methylation of mammalian DNA involves the addition of a methyl group to the 5’-carbon the cytosine in cytosine-guanine dinucleotides (termed CpG sites). This system of methylation likely evolved from a genomic defense system
responsible for preventing the spread of parasitic genetic elements. DNA methylation has since evolved to play an active role in maintaining genetic structure and genome regulation (Chen et al., 1998). Methylation is involved in X-chromosome inactivation (Mohandas et al., 1981; Csankovszki et al., 2001), silencing of transposable elements (Liu et al., 1994; Woodcock et al., 1997; Yoder et al., 1997; Walsh et al., 1998), tissue-specific gene expression (De Smet et al., 1996; De Smet et al., 1999; Jaenisch and Bird, 2003; Shiota, 2004), and gene imprinting (Bartolomei et al., 1993; Li et al., 1993; Stoger et al., 1993; Bird, 2002). DNA methylation is widespread throughout the genome, and the maintenance of methylation patterns is highly regulated and tissue-specific (Eckhardt et al., 2006; Illingworth et al., 2008). Patterns of methylation are established and maintained by DNA methyltransferase (DNMT) enzymes (Holli day and Pugh, 1975; Riggs, 2002; Jones and Liang, 2009). The absence of DNA methylation results in embryonic lethality, which highlights the crucial role of DNA methylation in development (Li et al., 1992; Okano et al., 1999; Goll and Bestor, 2005).

Methylation regulates pseudogenes within the genome (Grunau et al., 2000; Eckhardt et al., 2006). Pseudogenes are decayed copies of active genes that have arisen from either a duplication event in which the entire gene, or portion of a gene, is duplicated (non-processed pseudogenes) or from the retrotransposition of a gene transcript back into the genome (processed pseudogenes). An analysis of the human genome estimates that as many as 19,000 pseudogenes are evenly distributed throughout the genome, and approximately 70% of these are processed pseudogenes (Riggs, 2002; Rauch et al., 2009). Pseudogenes primarily arise from parent genes that are
transcriptionally active within the germ line, and 10% of genes within the human genome have at least one corresponding pseudogene (Riggs, 2002; Laurent et al., 2010).

The same regulatory network that inhibits transposable element movement likely induces DNA methylation on pseudogenes. Methylation of pseudogenes is elevated in embryos, likely as a mechanism for preventing the spread of retrotransposable elements during embryogenesis (Eckhardt et al., 2006; Zhang et al., 2006). In plants, the inactivation of methyltransferases resulted in the widespread activation of transposable elements and pseudogenes (Zhang et al., 2006), demonstrating that DNA methylation is sufficient to prevent the activation of pseudogenes. In humans, pseudogenes are highly methylated, presumably to prevent transposition (Eckhardt et al., 2006).

In order to better understand how methylation patterns are established and maintained on pseudogenes, we examined the methylation status of four pseudogenes derived from the translational enhancer LIN28. This gene is involved in early development and acts as a switch for developmental timing (Moss et al., 1997). LIN28 can also act as a reprogramming factor in the production of induced pluripotent stem cells (Yu et al., 2007; Darr and Benvenisty, 2009).

The protein-coding region of LIN28 contains a high concentration of CpG sites, making the gene a potential target for DNA methylation once inserted elsewhere in the genome as a pseudogene. LIN28 has given rise to 10 processed pseudogenes within the bovine genome that vary in length between 100 to 4000 bp. By measuring the methylation levels of selected pseudogenes and the gene of origin, we sought to determine whether the same regulatory mechanism that maintains methylation of the
LIN28 parent gene also controls the methylation status of LIN28 pseudogenes. Additionally we examined the expression of genes near the insertion site to determine whether pseudogene methylation is involved in transcriptional control of adjacent genes.

**Materials and Methods**

**Fibroblast Cell Culture**

Bovine fibroblasts isolated from skin were cultured in DMEM F12 (Thermo Scientific HyClone Laboratories, Logan, UT) supplemented with 15% fetal bovine serum (FBS) (Thermo Scientific HyClone Laboratories), 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were cultured at 37°C with 5% CO₂. For cell collection fibroblasts were treated with 0.25% trypsin prior to collection for RNA and DNA isolation.

**Oocyte Maturation**

Bovine ovaries were collected at a local abattoir (E.A. Miller, Hyrum, UT) and used for collecting oocytes. Oocytes with 3 to 8 mm follicles were aspirated along with cumulus complexes. Following aspiration, cumulus oocyte complexes were cultured at 37°C with 5% CO₂ for 18 to 22 hr in TCM 199 maturation medium containing 10% FBS (Thermo Scientific HyClone Laboratories), 0.05 mg/ml follicle stimulating hormone, 5 mg/ml luteinizing hormone, 100 U/ml penicillin, and 100 mg/ml streptomycin.

**In Vitro Fertilization**

Following 18 to 22 hr of oocyte maturation, cryopreserved bovine semen (Hoffman AI, Logan, UT) was thawed in a 37°C water bath. Live sperm were isolated by centrifugation through a 45%/90% percoll gradient. Sperm were suspended in Tyrode’s
albumin lactate pyruvate (TALP) and used for oocyte fertilization. Twenty-four hr post fertilization, cumulus cells were removed by vortexing the cumulus oocyte complex in phosphate buffered saline (PBS) containing 0.32 mM sodium pyruvate, 5.55 mM glucose, 3 mg/ml BSA, and 10 mg/ml hyaluronidase. Oocytes were washed through six drops of PBS and placed in co-culture dishes plated with cultured cumulus cells and cultured in CR2 medium.

**Tissue Collection and RNA Isolation**

Twenty-five pooled blastocyst embryos were collected after 8 days of culture in CR2 medium, snap frozen with liquid nitrogen, and stored at -80°C. Twenty-five pooled oocytes were collected after 22 hr of maturation, vortexed for 5 min in PBS to remove cumulus cells, snap frozen with liquid nitrogen, and stored at -80°C. RNA was isolated from oocyte and blastocyst embryo samples using the RNeasy Mini Kit (Qiagen, Germantown, MD) following manufacturer’s instructions. Bovine brain, liver, testes tissue samples were collected immediately after slaughter and suspended in RNALater (Ambion, Austin, TX). Samples were stored overnight at 4°C. Fibroblasts were collected as described above. RNA was isolated from brain, liver, testes, and fibroblasts using TRIzol reagent (Life Technologies, Carlsbad, CA). Tissue samples were homogenized in 3 ml TRIzol with a tissue homogenizer. Cells were incubated for 5 min at room temperature, combined with 0.6 ml chloroform, and mixed by inversion. Samples were centrifuged at 12,000 × g for 15 min at 4°C, the upper aqueous phase was removed and combined with 1.5 ml isopropyl alcohol, then centrifuged at 12,000 × g for 10 min at 4°C. Supernatant was removed and the RNA was washed with 75% ethanol
and centrifuged again, then dried and resuspended in H$_2$O. Isolated RNA was immediately converted to cDNA using the Superscript III Reverse Transcriptase Kit (Life Technologies) following manufacturer’s protocol. Samples were stored at -20°C until use.

**DNA Isolation and Bisulfite Conversion**

Twenty-five pooled oocytes and blastocyst embryos were snap frozen with liquid nitrogen and stored at -80°C until direct bisulfite conversion. DNA from brain, liver, testes, and fibroblast samples were collected from the interphase of the TRIzol treatment used for RNA collection. The collected interphase was combined with 0.9 ml ethanol, mixed by inversion, and centrifuged at 2000 × g for 5 min at 4°C. The pellet was suspended with 75% ethanol and washed by centrifugation three times. The DNA pellet was resuspended in 0.1 μM sodium citrate. The isolated DNA underwent bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA) according to manufacturer’s recommendation. DNA was stored at -20°C until use.

**Bisulfite PCR and 454 Sequencing**

Primers for bisulfite-converted DNA were designed for each pseudogene and the LIN28 parent gene. Primers covered CpG sites within and immediately surrounding the protein-coding sequence of LIN28. As the four examined pseudogenes maintain high sequence identity to the LIN28 parent gene and one another, primers were designed specific to each pseudogene to ensure only amplification of the target pseudogene. Bisulfite-converted DNA was used as a template for 25 μl PCR reactions using 1 to 3 μl
DNA. Reactions were carried out using the Mastercycler thermal cycler (Eppendorf, New York, NY). Primer concentrations were 0.6 µM for all reactions. All reactions were carried out using the following cycling parameters: 30 cycles of 94°C for 1 min, annealing temperature ranging from 54 to 59°C for 1 min, and 72°C for 1 min for 30 cycles. A second PCR was preformed using primers including the original primer sequence with the addition of the 454 adapter sequence, key sequence, and molecular identification tags (designated as N) to differentiate individual tissue samples (adapter A CGTATCGCCTCCCTCGCCATCAGNNNNNNNNNN attached to the forward primer and adapter B CTATGCGCCTTGCAGCCCGCTCAGNNNNNNNNNN attached to the reverse primer). The following cycling parameters were used: 94°C for 30 sec, annealing temperature ranging from 55 to 60°C for 30 sec, and 72°C for 30 sec for 15 cycles. The second reaction used 1 µl of PCR product from the first PCR and 0.3 µM primer. All PCR samples used GoTaq Green Master Mix (Promega, Madison, WI). All PCR reactions were carried out on a Mastercycler thermal cycler (Eppendorf). PCR reactions were purified with AMPure beads (Beckman Coulter, Brea, CA) and quantified using PicoGreen (Life Technologies). PCR product was sequenced using the 454 GD FLX Titanium DNA sequencer (Roche, Indianapolis, IN). Amplicon libraries were subjected to emulsion PCR to generate DNA-coated beads, loaded onto a PicoTiterPlate, and sequenced with a FLX Titanium DNA sequencing Kit according to the manufacturer’s protocol.
End-Point RT-PCR

End-point RT-PCR reactions targeting bovine cDNA were carried out using the Mastercycler thermal cycler (Eppendorf). GoTaq Green Master Mix (Promega) was used on all reactions with primer concentrations of 0.6 µM using the following cycling parameters: 40 cycles of 94°C 15 sec, annealing temperature ranging from 58 to 60°C for 15 sec, and 72°C for 30 sec for 35 cycles. Following cycling, samples were electrophoresed on a 1% agarose gel and imaged.

Sequence Analysis

Sequencing data was analyzed to generate the frequency of methylation using BISMA analysis software (Rohde et al., 2010). Data are expressed as the percentage of methylated CpG sites by normalizing methylation frequency data to the number of sequence reads obtained.

Results

We assessed the methylation status of four LIN28 processed pseudogenes in six bovine tissue samples, including brain, liver, testes, fibroblast cells, IVF blastocyst stage embryo, and oocyte. Three of the LIN28 pseudogenes contain the entire protein-coding sequence as well as transcribed regions up and downstream from the protein-coding region. These three pseudogenes are likely the products of retrotransposition of the LIN28 transcript following intron excision (Fig. 3-1). A fourth pseudogene contains only the terminal portion of the protein-coding region and downstream transcript (Fig. 3-1). These four pseudogenes were selected for examination based on their retention of the
protein-coding region, as well as insertion location and number of CpG sites. The pseudogenes examined were as follows: **LIN28P-Ch:3** (LOC784466) inserted into chromosome 3 approximately 20Kbp upstream of the UBQLN4 gene (84% identity with **LIN28**), **LIN28P-Ch:7** (LOC781442) on chromosome 7 inserted into the fourth intron of the **MAN2A1** gene (97% identity with **LIN28**) and **LIN28P-Ch:26** (LOC539705) on chromosome 26 inserted in the first intron of **ACADSB** (97% identity with **LIN28**) (Fig. 3-2). In addition, an identical pair of processed pseudogenes **LIN28P-Ch28** (LOC785096 and LOC785075) on chromosome 28 was included; these pseudogenes have no proximity to any gene (90% identity with the **LIN28** gene) (Fig. 3-2). The **LIN28P-Ch:28** pseudogenes likely arose from the insertion of a single pseudogene into chromosome 28, followed by a duplication event that generated a second copy of the initial pseudogene. The two pseudogenes are nearly 100% identical to one another, and differ by only three nucleotides and cannot be distinguished from one another. Primer amplification was indiscriminate of either pseudogene and analysis was carried out as though it were a single targeted site.

Primer sets were designed to profile each individual pseudogene for a sequence within the protein-coding region. Due to the high sequence identity between the pseudogenes and parent gene, the overlap of examined CpG sites was limited by our ability to amplify each individual pseudogene. Pseudogenes **LIN28P-Ch:3**, **LIN28P-Ch:7** and **LIN28P-Ch:26** all contain considerable overlap between identical CpG sites. **LIN28P-Ch:26** contains overlap of only two CpG sites with the **LIN28** parent gene. The **LIN28** parent gene also shares only three overlapping CpG sites with **LIN28P-Ch:28** (Fig.
Because of the restricted overlap of CpG specific sites, our assessment regarding differences between individual CpG dinucleotides was limited. Rather we focused our analysis to consider patterns of methylation among the amplified regions as a whole.

**Methylation Status of the LIN28 Family**

Our results indicate that LIN28 and all examined pseudogenes were methylated in all bovine cell and tissue types examined. Seven CpG dinucleotides within the LIN28 parent gene were analyzed. These dinucleotides are located in the third exon of LIN28. All of the CpG sites were methylated in each tissue to varying degrees (Fig. 3-3). In addition, all pseudogenes examined were methylated in all tissue samples. However, each pseudogene demonstrated a distinct methylation pattern that differs sharply from the LIN28 gene, and that correlates to the pseudogene insertion location.

The pseudogenes LIN28P-Ch:3 and LIN28P-Ch:28 were inserted into a location not associated with any gene, and both of these pseudogenes share a similar overall pattern of methylation (Fig. 3-3). We analyzed methylation at nine CpG dinucleotides within LIN28P-Ch:3 and eight CpG dinucleotides in LIN28P-Ch:28. In both pseudogenes, oocytes had the lowest levels of overall methylation in these pseudogenes with 52% average methylation across all CpG sites for LIN28P-Ch:3 and 41% for LIN28P-Ch:28. In contrast, average methylation was highest in blastocysts, at 81% for LIN28P-Ch:3 and 92% for LIN28P-Ch:28. The methylation patterns of both pseudogenes apparently deviated from patterns observed in the LIN28 parent gene. Within the LIN28 parent gene, oocytes had the highest frequency of methylation with an average of 93% across all CpG sites examined. Alternatively, the methylation of the
parent gene was lowest in blastocyst embryos, with only 56% of CpG sites carrying the methyl mark (Fig. 3-3). In LIN28P-Ch:28 we observed three overlapping CpG dinucleotides with the LIN28 parent gene. Although limited, within these three overlapping sites, the inverse pattern of methylation between the LIN28 parent gene and pseudogenes LIN28P-Ch:3 and LIN28P-Ch:28 is maintained.

In contrast to the tissue-specific methylation patterns, pseudogenes LIN28P-Ch:7 and LIN28P-Ch:26 both have a hypermethylated pattern. Both LIN28P-Ch:7 and LIN28P-Ch:26 were inserted into gene bodies. LIN28P-Ch:7 is inserted into the fourth intron of the gene MAN2A1. Over 90% of the 28 CpG sites inspected within this pseudogene were methylated in all tissue samples. The LIN28P-Ch:26 pseudogene lies within the first intron of the gene ACADSB and is 20 kbp from the gene IKZF5. As for LIN28P-Ch:7, all 28 CpG sites examined for the LIN28P-Ch:26 pseudogene were highly methylated at greater than 90% frequency (Fig. 3-3). The hypermethylation observed in both of these pseudogenes is in sharp contrast to the moderate levels of methylation of the LIN28 parent gene and pseudogenes not inserted into gene bodies, none of which were as consistently hypermethylated for all tissue samples.

These apparent differences in methylation correlate to the location of insertion of the pseudogene. LIN28 pseudogenes inserted into gene bodies were hypermethylated. Alternatively, LIN28 pseudogenes with an insertion location distant from a gene varied in the pattern of methylation in a tissue-specific manner, although these tissue-specific patterns were inverse with respect to the LIN28 parent gene (Fig. 3-3).
Single CpG Demethylation

Both LIN28P-Ch:7 and LIN28P-Ch:26 contain a single CpG dinucleotide that is characterized by a low frequency of methylation in a tissue-specific manner. The LIN28P-Ch:7 CpG dinucleotide at site 23 (Fig. 3-3) is under-methylated in three tissues. Methylation of this site is only 45% in the testes and blastocyst, whereas this site is devoid of any methylation in fibroblast cells. The same CpG dinucleotide 23 was additionally measured within the LIN28P-Ch:3 sequence, yet this site retained a high level of methylation for all tissues examined (Fig. 3-3).

LIN28P-Ch26 contains a similar CpG dinucleotide that is undermethylated in a tissue-specific manner at CpG site 16 in IVF blastocyst and oocyte samples. Both samples are hypomethylated with a 13% methylation frequency in IVF blastocyst and 5% frequency in oocytes, yet highly methylated for other tissue types (Fig. 3-3).

Expression of Genes Adjacent to LIN28 Pseudogenes

End-point RT-PCR was performed to examine expression of LIN28 and genes closely adjacent to the four LIN28 pseudogenes examined to determine whether methylation of pseudogenes correlated with differences in gene expression. Expression of the genes DHDDS, MAN2A1, ACADSB, and IKZF5 were measured in testes, liver, brain, fibroblast, IVF blastocyst, and oocyte samples (Fig. 3-4). DHDDS, MAN2A1, and ACADSB expression did not correspond to methylation patterns of adjacent pseudogenes (Figs. 3-3 and 3-4). IKZF5 is proximal to LIN28P-Ch:26, which does contain a tissue-specific methylation pattern of a single CpG site. Interestingly, the expression pattern for IKZF5 mirrors the pattern of methylation in that this gene is not expressed in oocytes or
blastocyst embryos (corresponding to samples with low methylation at site 16), but highly expressed in other tissue types (corresponding to samples with high methylation at site 16) (Figs. 3-3 and 3-4).

**Discussion**

The objective of our study was to determine whether the methylation of pseudogenes followed the same patterns as the functional parent gene by examining *LIN28* as a case study. An observation that the *LIN28* pseudogenes maintained an identical or highly similar methylation pattern as the *LIN28* parent gene would suggest that regulation of *LIN28* methylation was specific to the sequence of the gene and that methylation of pseudogenes with high sequence identity was maintained by the same mechanism(s) that maintained methylation of the *LIN28* parent gene. Alternatively, an observation that pseudogene methylation patterns deviated from the parent gene would indicate that pseudogenes are subject to local regulation of methylation patterns. In this study, we observed that *LIN28* pseudogenes do not recapitulate the same methylation status as *LIN28*, but rather appear to acquire methylation patterns independent of the parent gene. Furthermore, we observed that methylation levels of the examined pseudogenes correlate to the location of insertion. *LIN28* pseudogenes inserted into gene bodies were hypermethylated in all tissues examined. In contrast, pseudogenes inserted into genomic regions that are not proximal to genes had reduced overall methylation and the methylation pattern was dependent upon the tissue type. Additionally, pseudogenes not associated with genes had less methylation in tissue samples that were highly methylated in the parent gene.
We measured methylation of seven CpG dinucleotides located within the third exon of the \textit{LIN28} gene. Methylation of CpG dinucleotides within the gene body is generally associated with transcriptionally active genes (Rauch et al., 2009; Laurent et al., 2010). This pattern is in contrast to methylation of the 5’ upstream and promoter regions of genes, which are typically associated with transcriptional silencing. Methylation of these seven CpG dinucleotides was dependent upon tissue type. Notably, oocytes had the highest levels of methylation within the \textit{LIN28} parent gene. Oocytes generally maintain low levels of global methylation relative to somatic cells (Razin et al., 1984; Monk et al., 1987). Following fertilization, global methylation levels decline further and are then reestablished during embryonic and somatic cell development (Mayer et al., 2000; Oswald et al., 2000; Hajkova et al., 2002; Morgan et al., 2005). The methylation level observed in the \textit{LIN28} parent gene is counter to this pattern. However, methylation levels measured in the \textit{LIN28P-Ch:3} and \textit{LIN28P-Ch:28} pseudogenes are consistent with changes in methylation levels on a global level. Within both these pseudogenes, oocytes maintained low levels of methylation, and somatic cells maintained high levels of methylation. It is possible that the methylation patterns observed for \textit{LIN28P-Ch:3} and \textit{LIN28P-Ch:28} are maintained by the same mechanism that maintains global levels of methylation, while methylation of the \textit{LIN28} parent gene is maintained by a separate mechanism.

We also observed that methylation of pseudogenes depends on the genomic context into which the pseudogene was inserted. Both pseudogenes inserted into a gene, \textit{LIN28P-Ch:7} and \textit{LIN28P-Ch:26}, are hypermethylated in all tissues. On the other hand,
both pseudogenes not associated with a gene, *LIN28P-Ch:3* and *LIN28P-Ch:28*, vary in the pattern of methylation within different tissue samples, but tissue methylation levels were similar between the two pseudogenes. The similarity of methylation levels among all tissue types between similar pseudogenes suggests that pseudogenes share a common regulatory mechanism that establishes and maintains the methylation signature.

Within both pseudogenes inserted into gene bodies, we observed single CpG dinucleotides that exhibit demethylation in a tissue specific manner. Pseudogene *LIN28P-Ch:7* was inserted into the fourth intron of *MAN2A1*. Within the investigated CpG dinucleotides, a single CpG site at position 23 demonstrated a sharp reduction in methylation in the testes and blastocysts (both of which have 45% methylation), as well as fibroblasts (0% methylation). The same CpG site measured in *LIN28P-Ch:3* had no such reduction in methylation observed in any tissue studied, so it is likely that the apparent decline in methylation was specific to this pseudogene. This hypomethylated site exists within the context of three closely aligned CpG dinucleotides, both of which are highly methylated in all tissues. This site- and tissue-specific decrease in methylation points to a possible deliberate regulation of methylation at this locus that is unexplained in our study.

The *LIN28P-Ch:26* pseudogene is located within the first intron of *ACADSB* and 20 Kbp upstream from *IKZF5*. Within the *LIN28P-Ch:26* pseudogene, a single CpG dinucleotide at site 16 is hypomethylated in the oocyte and blastocyst samples, a sharp contrast to the hypermethylated status of all surrounding CpG dinucleotides as well as the same CpG site in the four remaining tissues. *IKZF5* was not expressed in either oocytes
or blastocyst embryos. Although the expression pattern of *IKZF5* was correlated with the tissue-specific methylation patterns observed for the pseudogene, it is doubtful that this single CpG site is involved in the transcriptional regulation of *IKZF5*. Although demethylation has been associated with transcriptional silencing (Hark et al., 2000; Lim and van Oudenaarden, 2007), the involvement of this specific CpG site in regulation of *IKZF5* is unlikely, as the site is removed from the transcriptional start site by 20kbp.

In conclusion, the four *LIN28* pseudogenes examined in this study maintained methylation patterns that differed from the parent gene and from one another according to their location of insertion. New knowledge on the regulation of pseudogenes via DNA methylation could contribute to greater understanding of the maintenance of global and/or regional patterns of methylation. Future work on this topic should focus on defining methylation patterns for other pseudogene families to determine whether all pseudogenes are maintained in a similar manner or whether sequence specific patterns can be identified through analysis of pseudogenes. Additionally, future studies should investigate expression of pseudogenes to determine how patterns of methylation correlate with transcript levels.

**References**


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Figure 3-1. **The ten processed pseudogenes of LIN28 aligned to the LIN28 processed transcript.** The horizontal filled in lines represent each pseudogene with the location relative to the start of the LIN28 protein-coding region indicated at the bottom and measured in base pairs. Vertical lines represents start and stop codons. Sequence identity of each pseudogene to the LIN28 parent transcript is indicated on the right column and the chromosome of insertion is indicated on the left. The dashed box shows the pseudogenes examined for methylation analysis.
Figure 3-2. *LIN28* pseudogene locations on each chromosome. Genes are represented by boxes with horizontal lines indicating approximate exon start locations. Pseudogene location is indicated by the darkened box. Arrows indicate gene transcription direction.
Figure 3-3. Heat map of LIN28 and LIN28 pseudogene methylation profile. (following page) LIN28 and pseudogenes include the examined tissues: testes, liver, brain, fibroblast, IVF blastocyst embryos, and oocytes. The total number of sequencing reads is indicated next to each sample. The average percentage methylation of CpG sites within the entire amplicon is shown for each pseudogene. Each heat map cell indicates the percentage of CpG sites positive for methylation. Red indicates high methylation and blue low methylation. Each CpG site corresponds to a number indicated above each sample that indicates the placement of each CpG site in relation to the protein-coding region. CpG sites of pseudogenes that do not correspond to a CpG location within the LIN28 parent gene are represented by ‘n’.
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|       | Liver      | 95      | 88 | 91 | 97 | 93 | 90 | 91 | 95 | 97 | 95 | 97 | 99 | 100| 96 | 93 | 100| 100| 97 | 95 | 98 | 67 | 94 | 95 | 96 | 95 |
|       | Brain      | 96      | 94 | 97 | 95 | 96 | 94 | 92 | 96 | 97 | 99 | 99 | 98 | 100| 98 | 88 | 100| 99 | 99 | 95 | 97 | 95 | 97 | 96 | 96 | 99 |
|       | Fibroblast | 92      | 95 | 98 | 97 | 94 | 86 | 94 | 97 | 97 | 96 | 100| 97 | 100| 94 | 89 | 99 | 100| 99 | 99 | 92 | 95 | 93 | 97 | 97 | 95 |
|       | IVF Blastocyst | 93      | 92 | 91 | 95 | 89 | 82 | 85 | 99 | 94 | 96 | 100| 98 | 100| 95 | 97 | 96 | 100| 98 | 99 | 96 | 99 | 99 | 97 | 93 | 45 |
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Figure 3-4. **End-point RT-PCR.** Expression of genes *LIN28, ACADSB, MAN2A1, DHDDS, IKZF5* and *ACTB* in the testes, liver, brain, fibroblast, IVF blastocyst embryos and oocytes.
CHAPTER 4

ACTIVATION OF THE P53 PATHWAY FOLLOWING BOVINE SOMATIC CELL NUCLEAR TRANSFER

Abstract

Embryos produced via somatic cell nuclear transfer have impaired development, reduced implantation rates, and have reduced viability as neonates. Failure of cloned organisms to thrive is largely believed to be the product of failed somatic cell reprogramming. Evidence points to a role for the P53 pathway in modulating reprogramming of the genome to a pluripotent state. The tumor suppressor P53 maintains genome integrity by activating apoptosis and inducing cell cycle arrest in response to cell stress or oncogene expression. Activation of the P53 pathway is contrary to the needs of a pre-implantation embryo, which should express numerous genes responsible for cell growth and proliferation. The aim of this study was to determine the extent of apoptosis in cloned bovine embryos throughout early development. Our results indicate that the P53 pathway is activated in cloned embryos. Additionally we observed that the cell cycle arrest activator $P21^{WAF1/Cip1}$ ($CDKN1A$) was commonly expressed in cloned embryos, suggesting that P53 also induces cell cycle arrest in SCNT embryos. Activation of the P53 pathway may be the result of upregulation of the reprogramming factors $C-MYC$, $SOX2$, $LIN28$, and $KLF4$, as all of these genes are upregulated in cloned embryos. These observations suggest that SCNT embryos are susceptible to blastomere depletion via apoptosis and cell cycle arrest in response to expression of reprogramming factors.
Introduction

Somatic cell nuclear transfer (SCNT) is an established method for the production of transgenic animals and reproduction of domestic species with phenotypically valuable traits. The procedure utilizes an enucleated oocyte as a recipient for a donor nucleus. Cloning has been successfully performed in a number of species. Nevertheless, the benefits of cloning are hindered due to the limited success SCNT development (Wells et al., 1999). Reconstructed SCNT embryos suffer from high rates of embryo failure and increased rates of fetal and neonate death. The success rate of reconstructed oocytes resulting in live births is less than 3% (Thibault, 2003), and the low success limits the widespread use of the technique. The low success rate is thought to be primarily the result of incomplete reprogramming of the donor cell genome (Wilmut et al., 1998; Colman, 1999; Kikyo and Wolffe, 2000; Solter, 2000; Dean et al., 2001; Humpherys et al., 2001; Reik et al., 2001; Niemann et al., 2008). However, studies also point to high numbers of apoptotic cells in embryos as another factor that contributes to preimplantation and early gestational failure (Fahrudin et al., 2002; Hao et al., 2003; Jang et al., 2004; Gjorret et al., 2005; Im et al., 2006; Kumar et al., 2007).

Programmed cell death by apoptosis is characterized by the morphological features of membrane blebbing, cell shrinkage, nuclear condensation, and DNA fragmentation (Kerr et al., 1972). Apoptosis plays an active role during early embryonic development (Jurisicova et al., 1998; Hardy et al., 2001; Liu et al., 2002; Neuber et al., 2002; Gjorret et al., 2003; Jurisicova et al., 2003). Within preimplantation embryos, apoptosis is responsible for the removal of blastomeres damaged by exogenous stress,
carrying genetic abnormalities or that have reduced developmental potential (Fabian et al., 2005).

Apoptosis in preimplantation embryos has been detected in multiple species and has been observed at higher frequency in embryos produced by SCNT than those produced by in vitro fertilization (IVF) (Fahrudin et al., 2002; Hao et al., 2003; Jang et al., 2004; Gjorret et al., 2005; Im et al., 2006; Kumar et al., 2007). Cloned blastocysts have lower overall cell numbers than IVF embryos, an observation consistent with elevated levels of apoptosis in SCNT embryos (Koo et al., 2000; Fahrudin et al., 2002; Gjorret et al., 2005; Terashita et al., 2011). The frequency of apoptotic cells and the cell count within preimplantation embryos are important indicators of developmental potential (Brison and Schultz, 1997; Brison and Schultz, 1998; Koo et al., 2000; Knijn et al., 2003). Embryos exposed to factors that increase apoptosis have reduced implantation rates and decreased fetal birth weight (Wuu et al., 1999). These observations suggest that increased apoptosis in early developing embryos can negatively affect post-implantation development.

Apoptosis is regulated by P53, which is activated by cell stress, DNA damage, and oncogene expression. Upon activation, P53 can induce pathways leading to senescence, cell cycle arrest, or apoptosis (Vousden and Lu, 2002). Upon activation, P53 translocates to the nucleus where it then induces expression of numerous genes that can either inhibit cell cycle progression or initiate programmed cell death. In the event of cell cycle arrest, $P21^{WAF1/Cip1}$ ($CDKN1A$) is transcriptionally upregulated by P53 (Brugarolas et al., 1999). P21 is an inhibitor of cyclin-dependent kinases (CDKs) that are required for
cell cycle progression. Alternatively, P53 can initiate programmed cell death by upregulating the expression of genes that initiate apoptosis. *BAX* is one such pro-apoptotic gene (Oltvai et al., 1993; Miyashita and Reed, 1995). BAX oligomerizes to form a channel on the mitochondrial membrane that induces the release of cytochrome c (Wei et al., 2001). In healthy cells, BAX is inhibited by BCL2, which forms a heterodimer with BAX (Yin et al., 1994). Under normal conditions, levels of BCL2 exceed BAX levels. This favors BCL2:BAX heterodimer formation and prevents activation of apoptosis. However upon upregulation of *BAX* by P53, BAX oligomers become common, allowing for activation of the intrinsic apoptotic pathway via release of cytochrome c from the mitochondria (Basu and Haldar, 1998).

Recently, the P53 pathway has been shown to modulate somatic cell reprogramming. Reprogramming is known to activate P53-mediated apoptosis and cell cycle arrest (Qin et al., 2007; Mali et al., 2008; Zhao et al., 2008). Knockout of P53 dramatically improves the efficiency of generating induced pluripotent stem cells (iPSCs) (Zhao et al., 2008). P53 null cell lines manifest dramatically higher reprogramming success rates (Hong et al., 2009; Kawamura et al., 2009). Several genes upregulated by P53 prevent survival of reprogrammed cells (Hong et al., 2009). Among these is *P21\textsuperscript{WAF1/Cip1}* \footnote{an observation that suggests cell cycle arrest is an additional consequence of somatic cell reprogramming. Cell lines that are recalcitrant to reprogramming can be successfully reprogrammed following P53 inhibition, providing further evidence for a role of P53 in somatic genome reprogramming (Utikal et al., 2009).}
The observations that P53 plays a role in modulating reprogramming somatic DNA to a pluripotent state for the generation of iPSCs has relevance for SCNT and the high rate of failure for SCNT embryos. The donor cell genome must undergo somatic cell reprogramming as a part of embryonic development. However, reprogramming the somatic genome may activate P53 and trigger either cell cycle arrest or apoptosis. P53 accumulation within IVF embryos has been observed to act as a direct impediment to embryo viability and implantation (Ganeshan et al., 2010). Decreased P53 expression led to significantly higher rates of fetal development following transfer of IVF embryos (Li et al., 2007). Furthermore, the number of apoptotic cells within an embryo was negatively correlated with the quality and overall viability of the embryo (Hao et al., 2004). Apoptosis has been observed to be higher in cloned embryos as measured by DNA degradation and morphological changes associated with apoptotic cells (Fahrudin et al., 2002; Hao et al., 2003; Jang et al., 2004; Gjorret et al., 2005; Im et al., 2006; Kumar et al., 2007). However, previous work associating SCNT with apoptosis have not measured the transcriptional activity of genes associated with the P53 pathway nor determined the cause of increased apoptosis following SCNT.

The aims of this study were to determine the extent of P53-mediated apoptosis within SCNT embryos and to ascertain the factors that activate apoptosis following SCNT. P53 expression can alter the developmental trajectory of SCNT preimplantation embryos and may function to remove inappropriately programmed or otherwise abnormal blastomeres from contributing to SCNT embryo development. Our findings indicate that
P53-mediated apoptosis is activated within SCNT embryos and that activation is likely in response to aberrant expression of reprogramming factors.

**Materials and Methods**

*Oocyte Maturation and In Vitro Fertilization*

Bovine ovaries were collected from a local abattoir (E.A. Miller, Hyrum, UT). Cumulus-oocyte complexes were isolated from 3 to 8 mm follicles by aspiration using an 18-gauge needle. TCM 199 maturation medium (Thermo Scientific HyClone Laboratories, Logan, UT) containing 10% FBS, 0.05 mg/ml follicle stimulating hormone, 5 mg/ml leutinizing hormone, 100 U/ml penicillin, and 100 mg/ml streptomycin was used to culture aspirated cumulus-oocyte complexes for 18 to 22 hr. Following oocyte maturation, cryopreserved bovine semen (Hoffman AI, Logan, UT) was thawed in a water bath, and live sperm were segregated through centrifugation in a 45%/90% percoll gradient. Collected live sperm were resuspended in Tyrode’s albumin lactate pyruvate (TALP) and used to fertilize matured oocytes. Twenty-four hr post-fertilization cumulus cells were removed from oocytes by vortexing in phosphate buffered saline (PBS) with 0.32 mM sodium pyruvate, 5.55 mM glucose, 3 mg/ml BSA, and 10 mg/ml hyaluronidase. Following vortexing, oocytes were washed in PBS and cultured in CR2 medium atop a layer of cumulus cells.

*Donor Cell Culture and SCNT Production*

Bovine fibroblasts collected from skin samples were cultured in DMEM F12 (Thermo Scientific HyClone Laboratories). Culture medium was supplemented with
15% fetal bovine serum (Thermo Scientific HyClone Laboratories), 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were seeded in a T25 culture flask and cultured at 37°C with 5% CO₂. Donor cells were collected prior to SCNT by treatment with 0.25% trypsin and resuspended in HEPES-buffered synthetic oviduct fluid. Oocytes had cumulus cells removed following maturation as described above. Oocytes in metaphase II were chosen for SCNT. Oocytes were enucleated and a single donor cell was transferred to the perivitelline space. Oocyte-donor cell fusion occurred in mannitol fusion medium (Wells et al., 1999) with two electric DC pulses of 2.2 kV/cm for 25 μsec. Following fusion, embryos were cultured in CR2 medium for 1 to 3 hr prior to activation, which was performed 23 to 25 hr post-maturation. SCNT embryos were activated with 5 min incubation in 5 mM ionomycin, followed by 4 hr incubation in 10 mg/ml cyclohexamide. SCNT embryos were placed in CR2 medium atop a layer of cumulus cells.

**Fluorescence Immunohistochemistry**

IVF and SCNT embryos were collected at the 8-cell and expanded blastocyst stages. Embryos were collected on three separate IVF/SCNT days. Embryos were incubated overnight at 4°C in fixative consisting of PBS with 4% formaldehyde and fixed overnight. Embryos were then washed at room temperature in PBS with 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO) (PBST) for 30 min, permeabilized at room temperature in PBS with 1% Triton X-100 for 30 min, and incubated overnight at 4°C in blocking buffer consisting of PBST with 1% BSA. Primary antibodies used were anti-CDK2 (ab79379), anti-P53 (ab80645) (Abcam, San Francisco, CA), anti- P21^{WAF1/Cip1}
Embryos were incubated with the primary antibody at 37°C for 3 hr, and then washed in PBST at room temperature for 30 min. Secondary antibodies used were: Alexa Fluor 594 IgG donkey anti-rabbit and Alexa Fluor 488 IgG donkey anti mouse (Life Technologies, Carlsbad, CA). Embryos were incubated with the appropriate secondary antibody at 37°C for 2 hr, and then washed in PBST at room temperature for 30 min. During each immunofluorescence analysis, two stage-appropriate IVF embryos were used as a negative control and incubated in the absence of the primary antibody, and underwent secondary antibody incubation. Following primary and secondary antibody incubation, embryos were incubated with Hoechst 33342 at room temperature for 20 min and then washed in PBST at room temperature for 10 min. Embryos were mounted onto slides in PBS with 70% glycerol and covered with a cover slip. Slides were imaged with a Zeiss Axiooobserver equipped with an Axiocam MRm camera.

*TUNEL Assay*

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was carried out using the Click-iT TUNEL Alexa Fluor Imaging Assay (Life Technologies) according to the manufacturer’s protocol. In brief, IVF and SCNT embryos were collected from a minimum of three separate IVF and SCNT production days and collected at the 8-cell and expanded blastocyst stages. Embryos were fixed in PBS with 4% formaldehyde at room temperature for 30 min and then permeabilized in PBS with 1% Triton X-100 at room temperature for 30 min. Embryos were then washed with PBST. Embryos were incubated in the TdT reaction cocktail to incorporate EdUTP
into dsDNA breaks. This was followed by incubation in Click-iT reaction cocktail to induce fluorescence of EdUTP. For each TUNEL assay two stage-appropriate embryos were used as a positive control by incubation in DNase I prior to performing the TUNEL assay. Additionally, two stage-appropriate IVF embryos were used as a negative control by incubation in the TdT reaction cocktail, but were withheld from Click-iT reaction cocktail. Following cocktail incubation, embryos were incubated with Hoechst 33342 and mounted to slides and imaged as described above. All blastomere nuclei positive for DNA degradation were counted in each embryo and the total number of apoptotic nuclei were divided by the total number of imaged embryos at the 8-cell (IVF, $N = 23$; SCNT, $N = 14$) and blastocyst (IVF, $N = 48$; SCNT, $N = 39$) stages to determine the average number of apoptotic blastomeres. A two-way ANOVA with Bonferroni post-hoc test for multiple comparisons was used to measure significance between stage-matched IVF and SCNT embryos.

**ROS Detection**

ROS detection was carried out using the Image-iT LIVE Green Reactive Oxygen Species Detection Kit (Life Technologies) following the manufacturer’s instructions. Briefly, embryos were washed in PB1+ (phosphate-buffered saline with Ca$^{2+}$ and Mg$^{2+}$ with 5.55 mM glucose, 0.32 mM sodium pyruvate, and 3 mg/ml BSA). Cells were labeled following manufacturer’s protocol. Embryos were stained with Hoechst and mounted to slides and imaged as described above.
Quantitative Reverse Transcriptase PCR (qRT-PCR)

Individual embryos were suspended in 3 µl H₂O, snap frozen in liquid nitrogen, and frozen at -80°C until reverse transcription. A total of 12 individual embryos were analyzed at the 2-cell, 8-cell, morula and blastocyst stages for both IVF and SCNT embryos. Reverse transcription was carried out using the Cellsdirect One-Step qRT-PCR Kit (Life Technologies) according to manufacturer’s protocol. Complementary DNA samples were immediately processed using the Biomark 96.96 Dynamic Array (Fluidigm, San Francisco, CA) using SYBR Green.

The delta-delta Ct method (Δ-ΔCt) was used for analysis of gene expression. The average Ct value for the housekeeping genes ACTB and GAPDH was subtracted from the Ct value of the gene of interest to yield a ΔCt value. Then, the average ΔCt value for each gene of interest for all embryos at each IVF stage was subtracted from the ΔCt of an individual embryo for each gene of interest to yield the Δ-ΔCt value. These Δ-ΔCt values were then used to calculate the expression level for SCNT embryos relative to IVF embryos at each developmental stage. A number of samples lacked a positive Ct value for certain genes. In order to calculate fold change any sample lacking a Ct value was assigned a Ct value of 40, the number of maximum cycles run. These assigned Ct values were used to generate fold change using the Δ-ΔCt method. Following fold change, all samples with failed reads were removed for expression data and statistical analysis. Any embryo that failed for the majority of PCR assays (i.e., no transcript detected for most genes in the array) or any PCR assay that failed in the majority of embryo samples (i.e.,
no transcript for a PCR assay for most of the embryo samples, IVF or SCNT) was excluded from further analysis.

**Statistical Analyses**

We employed a two-way ANOVA with Bonferroni post-hoc test for multiple comparisons (GraphPad Prism v 5.0, La Jolla, CA) to determine whether expression of a given gene was significantly different in SCNT embryos compared to their stage-matched IVF counterparts and to determine whether or not apparent differences in relative expression of a given gene in SCNT and IVF embryos were consistent across all embryo stages. A probability of \( P < 0.05 \) was considered significant.

**Results**

**Gene Expression in Cloned Embryos**

Gene transcription levels were measured for several apoptosis-specific genes using the BioMark Dynamic Array (Fluidigm). This method allowed the examination of relative gene expression levels for individual embryos. Figure 4-1 summarizes the expression data for all genes included in the Fluidigm array. An examination of expression patterns across individual embryos revealed high variability for nearly all genes assessed for both IVF and SCNT embryos. The gene set for this array included transcripts involved in early embryo development, pluripotency factors, genome reprogramming, apoptosis, cell cycle arrest, and several imprinted genes. For the purposes of this project, the analysis was focused on a subset of transcripts relevant to apoptosis and cell cycle arrest, as well as several pluripotency factors. Normalized gene
expression data and results of statistical analyses for the entire gene set are available in Figures 4-1 and Table 4-1, respectively.

Several genes relevant to the apoptosis pathway were frequently detected in SCNT embryos, but not expressed at measurable levels in IVF embryos. *BAX*, *BCL2*, and *P21*<sub>WAF1/Cip1</sub>, all of which play a role in regulating apoptosis or cell cycle arrest, are elevated in SCNT embryos compared to their IVF counterparts, although at specific developmental stages (Fig. 4-2). For example, *BAX* was expressed in 42%, 36%, or 58% of SCNT embryos at the 2-cell, 8-cell, and blastocyst stages, but not in morula SCNT embryos. Alternatively, fewer IVF embryos were positive for *BAX* expression at these developmental stages. Expression of *BAX* was comparatively higher in SCNT embryos than IVF at the 8-cell and blastocyst stages, although these differences were not statistically significant (Fig. 4-3, Table 2). *BCL2* is an anti-apoptosis protein that inhibits *BAX*, and the BCL2:BAX ratio is an important regulator of apoptosis. The *BCL2* transcript was detected in most embryos examined (Fig. 4-2), and levels of *BCL2* expression were significantly elevated in cloned blastocysts compared to IVF counterparts (*P* < 0.0001 by two-way ANOVA) (Table 4-2 and Fig. 4-3).

The *P21*<sub>WAF1/Cip1</sub> transcript was commonly detected in SCNT embryos at the 2-cell, 8-cell and blastocyst developmental stages, whereas this gene was infrequently detected in IVF embryos (Fig. 4-2). The most marked difference in relative expression was observed at the 8-cell development stage, where all SCNT embryos expressed comparatively higher levels of *P21*<sub>WAF1/Cip1</sub> than their IVF counterparts (*P* < 0.01, two-way ANOVA, Table 4-2).
Additional genes involved in apoptosis that are more commonly expressed in SCNT embryos included IGF2R, CX43, and PEG3 (Fig. 4-4). PEG3 and IGF2R were expressed at significantly higher levels in SCNT clones at the 2-cell or 8-cell stages respectively ($P < 0.01$ and $< 0.05$, respectively, two-way ANOVA, Table 4-2). CX43 transcript was detected in cloned embryos more frequently than IVF embryos at early developmental stages; an overall significant difference between IVF and cloned embryos was evident ($P < 0.05$), although the apparent levels of gene expression were not statistically different for any particular stage of development (Table 4-2).

Several genes that are involved in P53 activation or somatic cell genome reprogramming were also examined. Although not detected in any 2-cell or morula stage embryos, C-MYC was expressed in 73% of SCNT 8-cell embryos compared to only 9% for their IVF counterparts, and this expression was significantly higher ($P < 0.05$, two-way ANOVA, Fig. 4-5, Table 4-2). Similarly, LIN28 was more frequently expressed in cloned embryos compared to IVF (82% versus 9% for 8-cell; 50% versus 17% for blastocyst stage) (Fig. 4-5), expression levels were not significantly different between SCNT and IVF embryos at any of the developmental stages. SOX2 transcript was detected in most embryos without any apparent differences between embryos types (Fig. 4-5). However, levels of SOX2 expression were significantly higher in morula stage clones compared to IVF embryos ($P < 0.01$, two-way ANOVA, Table 4-2).

Finally, expression of two genes that may function to alleviate apoptotic pressure in SCNT embryos was examined. ETS2 can block activation of P53, and more SCNT embryos expressed ETS2 transcripts than did IVF embryos at the 8-cell and blastocyst
stages (Fig. 4-4); expression of ETS2 was significantly greater in blastocyst stage clones compared to IVF embryos ($P < 0.05$, two-way ANOVA, Table 4-2). Additionally, although KLF4 was not detected in any IVF embryos, about half of the SCNT embryos at the 8-cell stage expressed this gene at detectable levels ($P <0.0001$, two-way ANOVA, Fig. 4-5, Table 4-2).

**Fluorescence Immunohistochemistry**

Fluorescence immunohistochemistry was employed to examine protein expression for active P53, phosphorylated CDK2, P21, and BAX proteins in 8-cell and blastocyst stage IVF and SCNT embryos. The 8-cell stage was selected based upon the large number of genes that appeared differentially regulated at this critical developmental checkpoint; blastocyst stage embryos were assessed as this is the developmental stage at which embryos are evaluated prior to transfer for subsequent *in vivo* experiments.

Activated P53 accumulated within SCNT embryos to a greater extent than in IVF embryos at both the 8-cell and blastocyst stages (Fig. 4-6). Phosphorylated cyclin-dependent kinase 2 (phospho-CDK2) is a marker of cell cycle arrest. Immunostaining of phospho-CDK2 revealed higher levels in SCNT embryos at both the 8-cell and blastocyst stages (Fig. 4-6). Likewise, expression of P21 and BAX proteins were elevated in SCNT embryos at 8-cell and blastocyst stages (Fig. 4-7).

**TUNEL Assay**

The TUNEL assay was used to identify apoptotic cells in 8-cell and blastocyst stage SCNT and IVF embryos. Results of the TUNEL assay indicate that there is no
apparent difference in DNA degradation between IVF and SCNT embryos at the 8-cell stage (Fig. 4-8). However, at the blastocyst stage, SCNT embryos have significantly more apoptotic nuclei than do IVF embryos (Fig. 4-8). The mean number of apoptotic nuclei in IVF embryos was 3.9 in IVF compared to 6.8 in SCNT blastocysts. TUNEL measures DNA degradation in late-stage apoptotic cells, and the elevated incidence of DNA degradation in SCNT blastocysts confirms our expression and protein analysis of apoptotic factors.

*ROS Detection*

Reactive oxidative species (ROS) is an activator of apoptosis and may possibly induce apoptosis during preimplantation development. In order to determine if ROS was elevated in SCNT embryos and potentially responsible for activating apoptosis, we compared levels of ROS in SCNT and IVF embryos. ROS levels were highly variable among embryos, with no apparent patterns distinctive for SCNT or IVF embryos (data not shown).

*Discussion*

To determine whether the P53 pathway is activated in bovine cloned pre-implantation embryos, we compared gene and protein expression of key regulators of apoptosis, cell cycle progression and genome reprogramming in SCNT and IVF embryos. Herein, we report that a number of genes and proteins involved directly or indirectly in activation of P53 or subsequent cell cycle and apoptosis pathways are aberrantly expressed in SCNT embryos. We also report that apoptosis is elevated in blastocyst stage
bovine clones compared to their IVF counterparts. To our knowledge, this study is the first to employ the Fluidigm platform, which incorporates nanofluidics for nano-scale quantitative RT-PCR, to investigate gene expression in single bovine embryos. Results of these PCR analyses reveal a high level of inter-embryo variability in gene expression, an important observation when considering that success of SCNT is dependent on a single, high quality embryo. Collectively, the observations of this study point to the involvement of the P53 pathway in mediating apoptosis in SCNT embryos.

Apoptosis plays a key role in preimplantation development and is likely necessary to ensure the overall quality of blastomeres by eliminating defective cells from the embryo (Brison and Schultz, 1997; Hardy, 1997). Several studies have shown that apoptosis in SCNT embryos is elevated compared to embryos generated by IVF (Fahrudin et al., 2002; Hao et al., 2003; Jang et al., 2004; Gjorret et al., 2005; Im et al., 2006; Kumar et al., 2007). Additionally, SCNT embryos have a lower cell count compared to IVF embryos (Koo et al., 2000; Fahrudin et al., 2002; Gjorret et al., 2005; Terashita et al., 2011), which is likely the result of higher levels apoptosis in clones. Although apoptosis occurs in IVF embryos, it occurs at minimal levels, whereas apoptosis in cloned embryos increases throughout pre-implantation development (Hao et al., 2003).

P53 expression was elevated in SCNT embryos compared to their IVF counterparts, and this elevated expression may account for the lower blastomere count in clones observed in other studies (Devreker and Hardy, 1997; Byrne et al., 1999; Hardy et al., 2001) as well as the increased DNA degradation we observed in SCNT blastocysts in
this study. Although embryos with reduced cell counts are developmentally competent (Machaty et al., 1998), embryos with greater cell numbers are better prepared for implantation and developmental success (van Soom et al., 1997). Loss of SCNT embryos after transfer to surrogates and low pregnancy rates may be a consequence of high rates of apoptosis and reduced blastomere number during the preimplantation stage (Fahrudin et al., 2002).

\[ P21^{WAF1/Cip1} \] is transcriptionally regulated by P53 and is responsible for cell cycle arrest (Brugarolas et al., 1999). Upon transcriptional upregulation, P21 acts as an inhibitor of cyclin/CDK2 to block the progression of the cell cycle (Niculescu et al., 1998). Results of this study establish that \[ P21^{WAF1/Cip1} \] is expressed in a majority of SCNT embryos at the 2-cell, 8-cell, and blastocyst stage as measured by gene expression and protein immunofluorescence. As an additional measure of cell cycle arrest, we performed immunostaining of the phosphorylated form of CDK2, which is a regulator of cell cycle progression and is inhibited by phosphorylation (Morgan, 1995). CDKs are a target of inhibition by P21 (Poon et al., 1996), and phospho-CDK2 is higher in apoptotic cells. Our results indicate that phospho-CDK2 is prevalent in SCNT embryos, yet present at low levels in embryos produced via IVF. These results further support the role of cell cycle arrest as a factor that likely influences developmental competence of SCNT-derived embryos. While several research groups have examined the apoptotic status of SCNT embryos using the TUNEL assay and blastomere morphological changes (Hao et al., 2003; Jang et al., 2004; Gjorret et al., 2005; Im et al., 2006), this study was the first to investigate P53 activation as the trigger for apoptosis in SCNT embryos. Additionally,
cell cycle arrest cannot be detected using assays such as TUNEL. Our findings suggest that cell cycle arrest should be included as a standard in assessing SCNT embryo viability, as cessation of cell replication likely has grave consequences for development of SCNT preimplantation embryos. The arrested development of a blastomere at the 8-cell stage may reduce the total number of cells capable of contributing to embryo development. Indeed, the prior observations that SCNT embryo cell counts are comparatively low (Koo et al., 2000; Fahruadin et al., 2002; Gjorret et al., 2005; Terashita et al., 2011) may be the result of blastomere arrest at earlier embryo stages.

In addition to analyzing genes related to apoptosis, we also investigated reprogramming factors that may activate P53-mediated apoptosis. Overexpression of C-MYC has been shown to lead to the activation of apoptosis (Eischen et al., 1999; Pelengaris et al., 2002; Zhao et al., 2008; Kawamura et al., 2009). In this study, C-MYC was detected in a majority of 8-cell SCNT embryos, but in only a single IVF embryo. C-MYC may act as a trigger in activating apoptosis following SCNT. LIN28 was originally characterized as a heterchronic gene in C. elegans that acts as a switch that controls the timing and developmental fate of cells (Moss et al., 1997). LIN28 is a translational enhancer of C-MYC, and elevated levels of this gene, as observed in 8-cell clones, could contribute to the activation of P53-mediated apoptosis (West et al., 2009).

Expression of the pluripotency factor SOX2 was elevated in 2-cell and morula stage SCNT embryos compared to their IVF counterparts. This gene is critical during early embryo development and in somatic cell genome reprogramming (Takahashi et al., 2007; Yu et al., 2007; Silva et al., 2009). When expressed ectopically, SOX2 can induce
expression of $p21^{WAF1/Cip1}$ via the P53 pathway (Kawamura et al., 2009). Interestingly, in this study, the elevated expression of SOX2 appears to precede $P21^{WAF1/Cip1}$ expression in latter developmental stages. Although largely absent in most embryos examined, KLF4 expression was detected in more than half of the 8-cell cloned embryos examined. Because this protein has been shown to induce $P21^{WAF1/Cip1}$ in other studies (Zhang et al., 2000; Chen et al., 2003; Rowland et al., 2005), it stands to reason that KLF4 may have contributed to the higher levels of $p21^{WAF1/Cip1}$ expression also detected in 8-cell SCNT embryos in this study. Interestingly, while KLF4 can upregulate $P21^{WAF1/Cip1}$, it also functions as a repressor of P53 (Li et al., 2001; Rowland et al., 2005). Expression of C-MYC and KLF4 does not appear to be required for preimplantation development, as neither gene was detected with regular frequency in embryos produced via IVF. Both genes are expressed primarily at the 8-cell stage, and expression varied widely among SCNT embryos. It is possible that the activation of genes such as C-MYC, SOX2, KLF4, and LIN28 is a necessary step in donor cell reprogramming. However, the upregulation of these genes in SCNT embryos may contribute to activation of P53-mediated apoptosis and cell cycle arrest.

Our study also demonstrates that BAX protein expression is elevated in SCNT embryos at the 8-cell and blastocyst stages. Melka et al. (2010) observed higher expression of BAX in low quality embryos and suggested that BAX could serve as a marker for embryo quality in bovine embryos. BAX can be transcriptionally upregulated by P53 (Oltvai et al., 1993; Miyashita and Reed, 1995), or P53 can directly activate BAX in a transcription-independent manner (Schuler et al., 2000; Chipuk et al., 2005).
Whichever the mechanism, increased *BAX* expression is important for understanding SCNT embryo failure. It is however, essential to interpret *BAX* expression in context of *BCL2*. In bovine IVF development, embryos with high cytoplasmic fragmentation had elevated levels of BAX, whereas embryos with limited cytoplasmic fragmentation had low BAX and elevated BLC2 (McCurrach et al., 1997; Yang and Rajamahendran, 2002). BCL2 confers an anti-apoptotic response (Levine, 1997; Yang et al., 1997), but activated P53 can directly inhibit BCL2 to facilitate apoptosis (Wang et al., 1993; Chiou et al., 1994; Marin et al., 1994; Froesch et al., 1999). Analysis of *BCL2* transcription indicates that the gene is expressed within all embryos at all stages, but a statistically significant upregulation occurs within SCNT embryos at the blastocyst stage. *BAX* expression was observed in a subset of embryos and was more commonly expressed in SCNT embryos that IVF. Aberrant expression of genes involved in apoptosis was predominant in 8-cell SCNT embryos, which could induce a compensatory response to this apoptotic pressure leading to elevated expression of *BCL2* in blastocyst embryos as a mechanism to conserve blastomeres. This response may account for the development of SCNT embryos to the blastocyst stage despite the presence of apoptosis inducing factors such as *BAX*. Additionally, the *ETS2* gene is capable of suppressing P53. ETS2 acts as a proliferation factor that blocks apoptosis (Venanzoni et al., 1996; Sevilla et al., 1999). Indeed, the expression of *ETS2, KLF4*, and *BCL2* in clones may explain why some SCNT embryos are able to proceed through development and successfully develop despite activation of the P53 pathway.
In this study, we also detected elevated expression of *IGF2R, CX43, and PEG3* in a subset of SCNT embryos. *IGF2R* knockdown mice are typified by overgrowth during fetal development and perinatal death (Lau et al., 1994; Wang et al., 1994). Additionally, *IGF2R* has functions as a tumor suppressor, as loss of function mutations to this gene lead to tumor growth (De Souza et al., 1995; Hankins et al., 1996; Chappell et al., 1997; Ouyang et al., 1997; Piao et al., 1997; Wang et al., 1997; Xu et al., 1997; Yamada et al., 1997). During apoptosis, IGF2R acts as a death receptor through activation of mitochondrial-dependent apoptosis (Motyka et al., 2000). *IGF2R* overexpression increases cell susceptibility to apoptosis (Kang et al., 1999). Likewise CX43 contributes to apoptosis and is elevated in SCNT embryos. CX43 is a component of gap junctions and has been implicated in apoptosis (Huang et al., 2001; Kameritsch et al., 2013) and cell cycle arrest (Zhang et al., 2003). PEG3 facilitates the translocation of BAX to the mitochondria and participates in P53-mediated apoptosis (Deng and Wu, 2000). *PEG3* is expressed in all stages of embryo development, but is elevated in SCNT embryos at the 2-cell and morula stages. The elevated expression of *IGF2R, CX43, and PEG3* further highlights apoptotic environment under which SCNT embryos develop. Collectively, our observations point to a scenario in which SCNT embryos experience apoptotic pressure that may contribute to either activation of the programmed cell death pathway or cell cycle arrest. A consequence of either cell response would be the under-representation of the embryo cell population, placing that embryo at a selective disadvantage for robust placentation and fetal maintenance.
The TUNEL assay detects 3’-OH DNA strand breaks that occur during late-stage apoptosis (Gavrieli et al., 1992). TUNEL assay has been previously used to assess embryo viability during preimplantation development (Brison and Schultz, 1997; Byrne et al., 1999; Matwee et al., 2000; Gjorret et al., 2003; Gjorret et al., 2005). Earlier reports have shown that DNA fragmentation as measured by TUNEL is higher in SCNT embryos as compared to IVF (Fahrudin et al., 2002; Hao et al., 2003; Jang et al., 2004; Gjorret et al., 2005; Im et al., 2006), and our data is consistent with these findings. We observed no difference between IVF and SCNT at the 8-cell stage, which is expected as apoptosis related genes are minimally present at the 2-cell stage and onset of activation of the P53 pathway appears to occur at the 8-cell stage. Because TUNEL measures late-stage apoptosis, onset of apoptosis would not be expected until later in development, as we observed in blastocyst SCNT embryos.

The potential benefits of SCNT are currently hindered by the low success rate of the technique. A clearer understanding of the pathways implicated in the poor development of SCNT embryos may yield important knowledge that can be leveraged to develop interventions to overcome embryonic failure and improve developmental rates of cloned animals. Future studies should examine other downstream genes in the P53 pathway in order to gain additional insight regarding the mechanisms responsible for triggering P53-mediated apoptosis. We did not explore chromosomal aberrations within cloned embryos, which have been associated with reprogramming (Marion et al., 2009), and cannot rule out that increased apoptosis is the result of chromosomal anomalies. Inhibition of P53 during preimplantation development may give a developmental window
of opportunity in which blastomeres can proliferate and development of the embryo can occur prior to implantation.

References


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<tr>
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Table 4-2. Statistical Analysis of Gene Expression.

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Note: Fold changes were calculated using the Δ-ΔCt method using the averaged values of GAPDH and ACTB as an internal control. Individual fold changes for each IVF and SCNT embryos were measured against the mean IVF ΔCt at the corresponding embryo stage. The maximum Ct value of 40 was assigned to all embryos negative for gene expression in order to calculate fold change. Statistical analysis employed a two-way ANOVA with Bonferroni post-hoc test for multiple comparisons to measure significance between stage-matched IVF and SCNT embryos (Bonferroni post-hoc tests comparing IVF vs. SCNT column) as well as across embryo stages for both IVF and SCNT samples (Two-way ANOVA column). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Additional analyses were performed using the Student’s t-test with Welch’s correction compared IVF to SCNT embryos at each developmental stage (not shown); results of those analyses confirmed those obtained by the ANOVA shown here.
Figure 4-1. **Heat map of all genes analyzed for expression analysis.** Each cell of the heat map represents the relative expression of individual SCNT embryos and IVF embryos normalized to the mean expression for IVF at each developmental stage. The number of embryos included in the analyses is shown in parentheses. Colors represent the log_2 fold change for embryos positive for expression for each gene, with red indicating elevated expression and blue indicating low expression. Cells colored gray represent embryos for which the corresponding transcript was not detected.
**Figure 4-2.** Log$_2$ fold change expression values of *BAX*, *BCL2* and *P21*$_{WAF1/Cip1}$.
Open bars represent individual IVF embryos and solid bars represent individual SCNT embryos at the 2-cell, 8-cell, morula, and blastocyst stages. Fold change was calculated using the Δ-ΔCt method using the averaged values of *GAPDH* and *ACTB* as an internal control as described in Materials and Methods. Fold changes for individual IVF and SCNT embryos were calculated relative to the mean IVF ΔCt at the corresponding embryo stage. For those embryos with no transcript detection, a maximum Ct value of 40 was assigned in order to calculate the fold change. The percentage of embryos for which the indicated transcript was detected is shown below each Figure (N = 10 to 12 embryos).
Figure 4-3. **Average log₂ fold change expression values of BAX and BCL2.** Open bars represent individual IVF embryos and solid bars represent individual SCNT embryos at the 2-cell, 8-cell, morula, and blastocyst stages. Fold change was calculated using the ΔΔCt method using the averaged values of GAPDH and ACTB as an internal control as described in Materials and Methods. Fold changes for individual IVF and SCNT embryos were calculated relative to the mean IVF ΔCt at the corresponding embryo stage. For those embryos with no transcript detection, a maximum Ct value of 40 was assigned in order to calculate the fold change (N = 10 to 12 embryos).
Figure 4-4. Log₂ fold change expression values of *IGF2R, CX43, PEG3* and *ETS2*. (following page) Open bars represent individual IVF embryos and solid bars represent individual SCNT embryos at the 2-cell, 8-cell, morula, and blastocyst stages. Fold change was calculated using the Δ-ΔCt method using the averaged values of *GAPDH* and *ACTB* as an internal control as described in *Materials and Methods*. Fold changes for individual IVF and SCNT embryos were calculated relative to the mean IVF ΔCt at the corresponding embryo stage. For those embryos with no transcript detection, a maximum Ct value of 40 was assigned in order to calculate the fold change. The percentage of embryos for which the indicated transcript was detected is shown below each Figure (*N* = 10 to 12 embryos).
Figure 4-5. Log² fold change expression values of C-MYC, LIN28, SOX2 and KLF4. (following page) Open bars represent individual IVF embryos and solid bars represent individual SCNT embryos at the 2-cell, 8-cell, morula, and blastocyst stages. Fold change was calculated using the Δ-ΔCt method using the averaged values of GAPDH and ACTB as an internal control as described in Materials and Methods. Fold changes for individual IVF and SCNT embryos were calculated relative to the mean IVF ΔCt at the corresponding embryo stage. For those embryos with no transcript detection, a maximum Ct value of 40 was assigned in order to calculate the fold change. The percentage of embryos for which the indicated transcript was detected is shown below each Figure (N = 10 to 12 embryos).
**C-MYC**

- 2-Cell
- 8-Cell
- Morula
- Blastocyst

**LIN28**

- 2-Cell
- 8-Cell
- Morula
- Blastocyst

**SOX2**

- 2-Cell
- 8-Cell
- Morula
- Blastocyst

**KLF4**

- 2-Cell
- 8-Cell
- Morula
- Blastocyst
**Figure 4-6.** Representative immunofluorescence images for P53 and phosphorylated CDK2 in IVF and SCNT embryos. Alexa Fluor 488 detection of P53 (green) and Alexa Fluor 594 detection of phosphorylated CDK2 (pCDK2, red) are shown in 8-cell and blastocyst stage embryos. Nuclei were stained using Hoechst (blue). A merged image for all three fluorescence detection channels is shown. Images are representative of 12 to 18 embryos.
Figure 4-7. Representative immunofluorescence images for P21 and BAX in IVF and SCNT embryos. Alexa Fluor 488 detection of P21 (green) and Alexa Fluor 594 detection of BAX (red) are shown in 8-cell and blastocyst stage embryos. Nuclei were stained using Hoeschst (blue). A merged image for all three fluorescence detection channels is shown. Images are representative of 12 to 18 embryos.
Figure 4-8. Representative images and evaluation of apoptosis in SCNT and IVF embryos. (A) Cells undergoing apoptosis in 8-cell and blastocyst stage embryos were detected by TUNEL assay with Alexa Fluor 594 (red). Nuclei were stained using Hoeschst (blue). A merged image for both fluorescence detection channels is shown. Images are representative of embryos at the 8-cell (IVF, \(N=23\); SCNT, \(N=14\)) and blastocyst (IVF, \(N=48\); SCNT, \(N=39\)) embryos. (B) A bar graph indicates the average number of DNA fragmented nuclei per embryo at the 8-cell and blastocyst stages. Open bars represent IVF embryos and solid bars represent SCNT embryos. Data is expressed as mean and ± SEM. *, \(P<0.05\) indicates statistically significant difference between IVF and SCNT groups according to two-way ANOVA with Bonferroni post-hoc test for multiple comparisons.
CHAPTER 5

CONCLUSION

Over the past two decades, researchers have focused intensely on the problems associated with SCNT in an effort to improve the success rate of the technique. Several advances in the cloning procedure have been made, but have only led to modest improvements in success rates. Despite these collective efforts, SCNT remains inefficient, and the potential benefits from generating cloned offspring remain unrealized.

My dissertation research was concerned with determining the factors that affect SCNT embryo development. One line of inquiry of my work characterized the degree of apoptosis in SCNT embryos to determine whether programmed cell death is responsible for reduced embryo cell number and clone failure. We compared gene and protein expression of several genes involved in apoptosis in cloned embryos, as well as measured several factors that may play a role in activating apoptosis. Additionally, we assessed DNA degradation and confirmed that SCNT embryos have higher rates of apoptosis during preimplantation development. Results of these studies indicate that SCNT embryos have higher rates of apoptosis at the late developmental stage. These observations were confirmed by gene and protein expression of several apoptosis factors, including the apoptosis regulator P53, which was expressed at higher levels in SCNT embryos compared to embryos produced via IVF. Our results also showed upregulation of several genes that likely trigger apoptosis within SCNT embryos, including C-MYC, LIN28, SOX2, and KLF4. These findings help illuminate the cause of embryo failure and
may lead to interventions that increase the success rate for development of SCNT embryos by targeting the apoptosis pathway.

A second line of inquiry of my research was to determine whether the methylation signatures of the developmental genes POU5F1, NANOG, and SOX2 were appropriately reprogrammed in SCNT embryos prior to implantation. These genes are essential for early development, and the aberrant reprogramming of methylation patterns would likely lead to embryo degradation. Using bisulfite sequencing, we measured the methylation status of CpG islands associated with each gene and compared SCNT embryos to IVF and donor cell samples. We determined that specific CpG sites of all three genes were abnormally established prior to implantation. The genes NANOG and SOX2 contained CpG sites that were more similar to those of the donor cell than patterns observed in IVF embryos. POU5F1 also contained aberrant levels of methylation, but POU5F1 was hypomethylated relative to IVF embryos. Our results suggest that genes essential for development are aberrantly methylated, which may affect embryo developmental success.

One constraint of methylation studies is the limited data that can be obtained from experiments. The technique of choice for methylation analysis uses sodium bisulfite, a chemical that alters the sequence of DNA. Although the technique allows for single base pair analysis of a sequence of interest, sodium bisulfite degrades DNA and renders it unstable. Because of the problem of degraded DNA, amplified regions must be short, thus limiting the amount of data that can be generated during sequencing. These
problems are compounded when using preimplantation embryos as a source of DNA, as the low amount of starting material markedly limits the scope of experiments.

As few as five years ago, analysis of methylation required expansion of DNA fragments in bacterial colonies, followed by pyrosequencing; at most, researchers would gather 80 to 100 sequences for analysis. This limitation has recently been overcome with the advent of high-throughput sequencing technology. Next generation sequencing is contributing to more robust experiments and generating data sufficient to draw conclusions regarding the targeting and maintenance of methylation patterns. Such advances allow for more expansive data collection that can be applied to research questions involving cloned embryos. Most research in the area of DNA methylation has focused on methylation associated with CpG islands, but advances in the scope of data collection allow for the study of methylation in non-CpG island regions, including intragenic DNA. Knowledge of the methylation status of all CpG sites for the genome of a cloned embryo would facilitate a greater understanding of epigenetic reprogramming following SCNT.

A second challenge in methylation studies is the limited understanding of the biological consequences of DNA methylation. It is well understood that methylation of regions near transcriptional start sites is associated with transcriptional silencing, but the mechanism(s) directing the targeted methylation of specific genes or loci within a gene promoter region remain poorly understood. While experiments may reveal the pattern of methylation levels associated with genes, the elucidation of apparent differences in methylation status remains difficult due to a lack of knowledge about the interpretation of
this methylation code by transcription regulators. For example, do specific methylation sites direct activation of transcription or is regional methylation critical for a specific gene? Is there a necessary threshold of CpG methylation frequency within a regulatory region? Without a better understanding of the mode of silencing following DNA methylation, interpretation of results is limited.

In order to address some of these problems and to expand our knowledge regarding how methylation targets and maintains a pattern within cells, we characterized the methylation pattern of a pseudogene family in order to assess how the genome treats identical sequences of DNA. We examined four pseudogenes derived from the LIN28 gene, as well as methylation of the parent gene itself. Results of these analyses showed that pseudogenes are not methylated with the same pattern as the parent gene, but rather maintain methylation levels specific to the region into which the pseudogene was inserted. These findings indicate that methylation maintenance enzymes are targeted to different genomic regions within the cell, and may ultimately lead to greater insights regarding the methylation machinery.

The research reported in this dissertation has added to the body of knowledge of the mechanisms of methylation regulation and helps to answer the question of why SCNT embryos fail. Understanding the role of apoptosis in SCNT development may lead to better procedures, culture conditions or treatments that will better enable embryos to develop following SCNT. Targeted disruption of the apoptosis pathway may yield improvements in the cloning procedure. If apoptosis could be briefly abated during preimplantation development, SCNT implantation and birth rates may increase.
However, it is possible that apoptosis functions to selectively remove dysfunctional or damaged blastomeres from the developing embryo and that inhibition of this process could allow for the inappropriate expansion of aberrant cells. Additionally, by characterizing the reprogramming inefficiencies of SCNT embryos, this work contributes to the body of knowledge of epigenetic regulation. Identifying epigenetic factors that may be responsible for reduced embryo viability and introducing information that suggests how methylation occurs differently in different regions of DNA may reveal better procedures to partially reprogram donor cells prior to nuclear transfer. Continued research based on these conclusions may lead to more effective procedures for SCNT and greater success of the technique.
Curriculum Vitae

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Publications


Publications in Preparation
Davis AP, Benninghoff AD, Sessions BR, Meng Q, Thomas AJ, White KL. (In preparation) DNA methylation reprogramming of the POU5F1, NANOG, SOX2 and KLF4 genes following somatic cell nuclear transfer. Mol Repro Dev


Davis AP, Meng Q, Sessions BR, White KL, Benninghoff AD. (In preparation) Activation of the P53 pathway following somatic cell nuclear transfer. Reproduction

Abstracts
Davis AP, Benninghoff AD, Meng Q, Sessions BR, Thomas AJ, White KL. Activation of the P53 apoptosis Pathway following somatic cell nuclear transfer. Presented at the ADVS Graduate Student Symposium. August 6, 2012. Logan, UT.


**Invited Presentations**

Davis AP, White KL. Sodium bisulfite sequencing shows aberrant epigenetic reprogramming following somatic cell nuclear transfer. Presented at the Center for Integrated BioSystems Research Seminar. November 2008 Logan, UT.