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Characterization of porcine adipose tissue-derived stem cells

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CHARACTERIZATION OF PORCINE ADIPOSE TISSUE-DERIVED STEM CELLS

A Dissertation

**Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy**

in

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

by

Kellie J. Williams

M.S., University of Oklahoma Health Sciences Center, 2005

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LIST OF ABBREVIATIONS

NT.....	Nuclear Transfer
pASC.....	Porcine Adipose tissue-derived Stem Cells
MSC.....	Mesenchymal Stem Cells
DNA.....	Deoxyribonucleic Acid
FPF.....	Fetal Porcine Fibroblast
PD.....	Population Doubling
LOS.....	Large Offspring Syndrome
ECL.....	Enhanced Chemiluminescence
mRNA.....	Messenger Ribonucleic Acid
cDNA.....	Complementary DNA
PCR.....	Polymerase Chain Reaction
qPCR.....	Quantitative PCR
ICM.....	Inner Cell Mass
POH.....	Progressive Osseous Heteroplasia
HSC.....	Hematopoietic Stem Cell
CFU-f.....	Colony-forming Unit-fibroblastic
LIF.....	Leukemia Inhibitory Factor
Shh.....	Sonic Hedgehog
EGF.....	Epidermal Growth Factor
FGF.....	Fibroblast Growth Factor
TGF.....	Transforming Growth Factor
Dnmt.....	DNA Methyltransferase
HAT.....	Histone Acetyltransferase
HDAC.....	Histone Deacetyltransferase

H3.....	Histone 3
miRNA.....	Micro RNA
priRNA.....	Primary miRNA
BMP.....	Bone Morphogenic Protein
GDF.....	Growth and Differentiating Factor
MAPK.....	Mitogen-activated Protein Kinase
PPAR γ	Peroxisome Proliferator-activated Receptor
TNF.....	Tumor Necrosis Factor
RXR.....	Retinoid X Receptor
C/EBP.....	CCAAT/Enhancer Binding Protein
SCD.....	Stearoyl CoA Desaturase
GLUT.....	Glucose Transporter
PEPCK.....	Phosphoenolpyruvate Carboxykinase
TIP.....	Tension-induced/inhibited Protein
WAT.....	White Adipose Tissue
BAT.....	Brown Adipose Tissue
POU.....	Pit/Oct/Unc
HMG.....	High Mobility Group
dPBS.....	Dulbecco's Phosphate Buffered Saline
P/S.....	Penicillin/Streptomycin
DMEM.....	Dulbecco's Modified Eagle Medium
FBS.....	Fetal Bovine Serum
BSA.....	Bovine Serum Albumin
ChIP.....	Chromatin Immunoprecipitation

ABSTRACT

Nuclear transfer (NT) results in relatively low success rates and research has reported substantial problems with the welfare of the cloned animals produced. Evidence suggests that the use of a less differentiated donor cell, such as a stem cell, may lead to an increase in the efficiency of the NT procedure. To this end, stem cell characteristics must be clearly defined in order to isolate, identify and optimally culture potential donor cells. Stem cell characteristics such as self-renewal and differentiation have not been identified in stem cells from porcine adipose tissue, an easily attainable tissue resource. At this time, the phenotypic profile of cell surface proteins and chromosomal stability of porcine adipose tissue-derived stem cells (pASCs) through *in vitro* culture have not been described. In addition, research has not identified epigenetic modifications in adult somatic mesenchymal stem cells (MSCs) from porcine adipose tissue.

The objective of Project 1 was to develop a protocol for the isolation and culture of pASCs and to determine stem cell-like characteristics. The objective of Project 2 was to partially characterize the surface protein phenotype of undifferentiated porcine ASCs and to determine the chromosomal stability through *in vitro* culture. Research has indicated that the inefficiency of NT may be attributed to changes in DNA methylation and histone acetylation as a result of the duration of somatic cell *in vitro* culture. The objective of Project 3 was to analyze pASCs and compare them to fetal porcine fibroblasts (FPF) for gene expression profiles of chromatin remodeling proteins, global methylation and acetylation patterns and methylated lysine residues within promoter regions of developmentally important transcription factors.

Improved culture conditions and defined cellular characteristics of these pASCs have been identified. pASCs can self-renew and differentiate into multiple tissue lineages. Porcine ASCs do not express the stem cell surface markers analyzed at similar levels compared with those of human ASCs reported in the literature. Also, *in vitro*-cultured porcine ASCs used as donor cells for NT should be chosen from early population doublings (PDs) due to increased

levels of aneuploidy at later PDs. Further characterization of porcine ASCs in this manner could assist the isolation and purification of a population of MSCs from the easily obtainable tissue of adipose that can later be tested as donor cells in nuclear transfer.

CHAPTER I

INTRODUCTION

Currently, nuclear transfer (NT) results in relatively low success rates of approximately 1-2% (Hiendleder et al., 2005) and research has reported substantial problems with the health and welfare of the cloned animals. Developmental abnormalities observed following NT define Large Offspring Syndrome (LOS) and include low pregnancy rates, high percentages of loss during early and late pregnancy, stillbirths, early postnatal deaths, short life-span, obesity and malformations (Young et al., 2001). LOS may be caused by several factors such as using an inappropriate donor cell and/or recipient oocyte, the inability to properly synchronize the cell cycle phase of the donor nucleus and the recipient cytoplasm and inadequate reprogramming of the donor genome. Also, the inappropriate handling of oocytes, somatic cells and embryos during maturation with the subjection of these cells to the various necessary manipulations and cultural techniques could result in cellular stresses leading to LOS.

Successful NT in combination with applicable genetic modifications must be conducted using normal diploid cells before they reach senescence (Denning et al., 2001). Primary cells utilized to establish cell culture possess the capacity to survive only a finite number of population doublings (PDs) before cellular senescence is reached (Hayflick and Moorhead, 1961). As cells approach this limit, the rate of proliferation decreases (Cameron, 1972) and the probability of cells to undergo cytokinetic abnormalities and chromosomal alterations increases substantially (Giraldo et al., 2006; Oback and Wells, 2002). The current techniques employed today to establish and maintain primary *in vitro* cell cultures may also be responsible for the induction of these anomalies (Chu, 1962; Denning et al., 2001; Kasinathan et al., 2001; Oback and Wells, 2002; Rubin, 1997). Spontaneous transformations, which will also influence the success of NT, have been described in human and murine adipose tissue-derived mesenchymal stem cells (MSCs) (Rubio et al., 2005) and have been reported to accumulate proportionally to the time in culture (Obach and Wells, 2002). However, other studies indicate

that human bone marrow and adipose tissue-derived MSCs do not mutate and maintain chromosomal configuration through culture (Bernardo et al., 2007; Meza-Zepeda et al., 2008).

Chromosomal aberrations and a limited proliferative capacity of somatic donor cells cultured *in vitro* dramatically influence the success of NT (Kasinathan et al., 2001). The manifestation of genetic modifications in donor cells for NT requires a significant number of PDs and, unfortunately, these deleterious effects are impossible at this time to circumvent. But the identification of a donor cell that is genetically stable for a long period of time *in vitro* may be the key to increasing the efficiency of NT.

Stem cell biology is one of the most active areas in biomedical research today. However, there is much controversy surrounding the use of human embryonic stem cells in research for medical applications. This controversy has spurred the focus of research to the isolation of alternative stem cell sources. Diligent efforts in current research are focusing on the isolation and characterization of adult stem cells. Adult stem cells have been isolated from many different tissues including blood, fat, bone marrow, organs such as the liver and retina, and in cord blood. Preliminary evidence suggests that these cells may be useful in clinical applications such as tissue engineering and cord blood transplants.

MSCs are pluripotent precursors meaning that they can differentiate into any cell type except for a totipotent cell (which can differentiate into all cell types). MSCs can be found in adult tissues such as adipose tissue, bone marrow (BM), and cord blood (CB). MSCs support differentiation into adipocyte, osteoblast and other mesodermal cell types. Depending on the culture conditions, MSCs can be cultured *in vitro* as pluripotent cells or differentiated cells with a fibroblastic morphology. Gene and protein expression profiles have been defined in some species specific MSCs showing the presence of cell surface markers such as CD34 and CD44.

At this point, knowledge of the optimal culture conditions for pluripotent and differentiated MSCs from adipose, bone marrow and cord blood is limited. Also, culture characteristics of these cells have not been clearly defined and studies generating gene and

protein expression profiles are underway but still remain inconclusive. Currently, nuclear transfer utilizing a true MSC as the donor cell is unconvincing most likely due to inefficient isolation/purification protocols and characterization. Therefore, it is important that the true nature and potential of these cells be defined in order to best apply them to the medical field. Also, in lieu of the controversy associated with embryonic stem cells, an animal model must be developed to maintain the progress of research in this area.

The inherent ability of mesenchymal stem cells such as porcine adipose tissue-derived stem cells (pASCs) to proliferate *in vitro* and maintain their aptitude for pluripotency (Williams et al., 2008) makes them attractive candidates as donor cells for more efficient reprogramming during NT compared to somatic cells currently in use. Studies have however generated contradicting results for the success of stem cells in NT; for example, skin-derived stem cells increased NT efficiency (Hao et al., 2009; Zhu et al., 2004) while hematopoietic stem cells did not (Inoue et al., 2006; Sung et al., 2006). Porcine ASCs have not been tested as donor cells for NT at this time.

Multiple different research groups have characterized human ASCs by describing the surface phenotype, however, there are many subtle discrepancies represented throughout the literature. The effects of these differences on the identity of these cells remain unknown. Various factors may play a role in producing these inconsistencies. For example, porcine ASCs are isolated from fat depots likely different from human ASC liposuction aspirates. Also, the age of the donor may be different and cause the results to disagree. In addition, isolation procedures used, the specific cell seeding density, differences in culture media used, culture duration, the cell cycle, the passage numbers analyzed, whether the cells were cryopreserved prior to analysis and even the exposure to plastic all may cause incongruent results. Therefore, it is important to characterize porcine ASCs on the cell surface level.

NT results in relatively low success rates and research has reported substantial problems with the welfare of the cloned animals produced. Evidence suggests that the use of a

less differentiated donor cell may lead to an increase in the efficiency of the NT procedure. To this end, stem cell characteristics must be clearly defined in order to isolate, identify and optimally culture potential donor cells. Stem cell characteristics such as self-renewal and differentiation have not been identified in stem cells from porcine adipose tissue, an easily attainable tissue source. At this time, the phenotypic profile of cell surface proteins and chromosomal stability of pASCs through *in vitro* culture has not been described. In addition, research has not identified epigenetic modifications in adult somatic MSCs from porcine adipose tissue.

The objective of Project 1 was to develop a protocol for the isolation and culture of pASCs and to determine stem cell-like characteristics. Primary cultures were established and cell cultures were maintained. Cloning capacity was determined using a ring cloning procedure. Primary cultures and clones were differentiated and stained for multiple differentiated phenotypes. An average of 2,700,000 nucleated cells/ml was isolated, 26% were adherent, and cells completed a cell cycle approximately every 3.3 d. Ring cloning identified 19 colonies. Primary cultures and clones were determined to differentiate along osteogenic, adipogenic and chondrogenic tissue lineages. Improved culture conditions and defined cellular characteristics of self-renewal of these pASCs have been identified. Porcine ASCs can self-renew, can differentiate into multiple tissue lineages and they express CD34.

The objective of Project 2 was to partially characterize the surface protein phenotype of undifferentiated porcine ASCs and to determine the chromosomal stability through *in vitro* culture. Cell surfaces were immunolabeled for stem cell surface proteins and analyzed by flow cytometric analysis as well as by western blot analysis paired with an enhanced chemiluminescence (ECL) kit. Metaphase spreads were prepared for each cell line and counted to determine chromosomal stability through *in vitro* culture. Cells at early and late PDs expressed CD29 and CD90. All other markers analyzed were expressed at low levels, including CD3, CD8, CD44, CD117 (c-Kit), SSEA-4 and Sca-1. Of 714 metaphases analyzed 71.3%

were aneuploid. Cells cultured through early PDs (PD 1-10) had a significantly lower percentage of aneuploidy compared to cells at late PDs (PD 11-20 and PD 21-30). Porcine ASCs do not express the stem cell surface markers analyzed at similar levels compared with those of human ASCs reported in the literature. Also, *in vitro* cultured porcine ASCs used as donor cells for NT should be chosen from early PDs due to increased levels of aneuploidy at later PDs.

Research has indicated that the inefficiency of NT may be attributed to changes in DNA methylation and histone acetylation as a result of the duration of somatic cell *in vitro* culture. The objective of Project 3 was to analyze pASCs alongside fetal porcine fibroblasts (FPF) for gene expression profiles of chromatin remodeling proteins and global methylation and acetylation patterns and to determine the presence of bivalent domains harboring both histone H3 lysine 4 and 27 tri-methylation within the promoter regions of developmentally important transcription factors. Cells were immunolabeled with anti-5-methylcytidine and anti-acetyl histone H3 and analyzed by flow cytometric analysis. Messenger RNA (mRNA) was isolated and reverse transcribed into complementary DNA (cDNA). Quantitative PCR (qPCR) was performed and a mean fold change in transcript expression was determined. Chromatin immunoprecipitation assay coupled with a qPCR was used to determine the presence of the bivalent domains. pASCs demonstrated a consistently lower level of DNA methylation and histone acetylation through passages 2 through 7; whereas, the patterns for FPFs varied. The expression levels of chromatin remodeling transcripts remained lower in pASCs throughout culture when compared to FPFs. pASCs possess the bivalent chromatin structure within the promoter region of the developmentally important transcription factor OCT-4. Further characterization of porcine ASCs in this manner could assist the isolation and purification of a population of MSCs from the easily obtainable tissue of adipose that can later be tested as donor cells in nuclear transfer.

Cutting edge methods for producing transgenic animals still need much improvement considering the inefficiency and expenses involved. Researchers in industry are engineering bacteria or mammalian cells into bioreactors for producing recombinant proteins for therapeutic treatments. However, these tiny bodies are incapable of producing the necessary mass quantity to adequately service the growing demand. The current shortfall hinders such therapeutic products from entering the marketplace thereby preventing patient access to new therapies, which in turn denies revenue to pharmaceutical companies who have invested large sums of money in product development.

By employing cells such as somatic stem cells or cells demonstrating stem-like characteristics as donor cells for nuclear transfer, resultant anomalies such as those which classify LOS may be averted and increase the success rates of nuclear transfer. An increase in the efficiency of transgenic animal production will provide a substitute factory for the manufacture of these recombinant proteins and offer a more cost effective mechanism for the production of large quantities (i.e., kilograms). An increased efficiency will also shorten the time required to produce an adequate number of live transgenic offspring for pharmaceutical and other biomedical purposes and therefore decrease the ultimate cost of production. In turn, this will eliminate economic barriers currently blocking the application of this technology from commercial large-scale production of transgenic animals and therefore make transgenic animals a more appealing method of producing recombinant proteins.

Controversy associated with the use of human tissues necessitates the development of an animal model to continue research in this important area. Many treatments have already resulted from the use of tissues generated through advancements made in the porcine animal model. Therefore, the improvement of culture conditions and the definition of cellular characteristics for the efficient isolation and purification of a population of MSCs from porcine tissues to be used as donor cells in nuclear transfer will create an important animal model for additional future biomedical applications.

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CHAPTER II

LITERATURE REVIEW

Clinical Applications of Stem Cell Biology

The potential impact of the field of regenerative medicine has a multitude of applications through stem cell biology. The expansion of our basic knowledge of stem cell biology will greatly impact scientific areas including development, cancer, and aging as well as provide a foundation for the development of tissue engineering (Merok and Sherley, 2001). An array of scientific disciplines have joined efforts to develop the techniques involved in tissue engineering for repairing, replacing, or regenerating diseased tissues and organs. With the advancement of this biotechnology, for example, scaffold biomaterials can be combined with a variety of stem cells to treat disease through cell-based therapies. Other cell therapeutic applications include bone marrow transplants, cartilage, tendon, ligament, and bone repair, treatment of vascular disease and even the production of transgenic animals (Merok and Sherley, 2001).

Several applications involve the technique of cell transplantation. Embryonic stem cells have the capability to form biologically functional cell populations suitable for integration, response and function within a diseased tissue. Embryonic stem cells produce these cell populations in response to signaling pathways induced by factors such as cytokines, transcription factors and cell-to-cell interactions. Multiple cell populations derived from embryonic stem cells, including cardiomyocytes (Klug et al., 1996), insulin-secreting cells (Soria et al., 2000) and neuronal precursor cells (Brustle et al., 1999; McDonald et al., 1999) have been successfully integrated within the host tissue and displayed the appropriate cell functions. For example, upon transplantation into the spinal cords of myelin-deficient rats, glial precursor cells derived from embryonic stem cells successfully differentiated into astrocytes and oligodendrocytes capable of forming myelin at the site of injection and in migration several millimeters away from the injection site (Brustle et al., 1999; Liu et al., 2000).

Candidate cells for such promising applications must meet several requirements to be eligible for classification as a stem cell population. Cells must have the capacity for self-renewal, must have long-term viability and must have the ability to give rise to differentiated cell types to be considered “stem cells.” The isolation and culture of large numbers of cells that will uniformly differentiate to a functional state, such as those embryonic stem cell-derived glial precursors, is necessary to gain eligibility for application to cell therapies (Tsai et al., 2002).

Stem Cell Definition

In 1855, Franz von Leydig postulated that life did not begin in a spontaneous fashion but rather from preexisting life. In agreement with his hypothesis, Rudolf Virchow developed a theory called *omnis cellula e cellula* meaning that all cells within an organism arise from preexisting cells (Oberling and Woglom, 1944). With these concepts, scientists began describing stem cells with the understanding that all cells are derived from the fertilized ova. For example, Edmund Beecher Wilson began describing mitotically quiescent primordial germ cells in 1896 (Wilson, 1896). Collectively, these pioneering minds initiated the standing concept that a stem cell is the functional unit of life that gives rise to progeny cells that can differentiate into the cells of the various tissues that make up an organism.

Stem cells are genetically identical to the stem cell from which it was derived, that is to say stem cells are clonogenic in nature. Theoretically, a stem cell can undergo an infinite number of repeated cell divisions for many generations without differentiating into a specialized cell of a certain tissue or function. During early embryonic development, cell division gives rise to two daughter cells each with a full chromosome complement and the capability of forming all differentiated cell types. Division in this manner is referred to as symmetric division (Merok and Sherley, 2001). Adult stem cells, or those stem cells derived from adult tissues, demonstrate asymmetric division which yields two daughter cells including one cell that remains a progenitor cell and one cell that begins the process of terminal determination or differentiation (Merok and Sherley, 2001).

Again, stem cell requirements include the capacity for self-renewal, long-term viability and the ability to give rise to differentiated cell types. Differentiation of stem cells into specialized precursor cell types will commence as a result of inherent genetic characteristics and in response to multiple environmental cues. Various cytokines, transcription factors, growth factors and cell-to-cell interactions dictate the fate of cell commitment. In 1974, Friedenstein determined that exposing cells to increasing amounts of serum fibroblast growth factor-2 caused the cells to initiate cell cycle activity (Friedenstein et al., 1974). In addition to growth factors, a lower seeding density can increase the expansion rate of cells *in vitro* as well as increase differentiation potential (Sekiya et al., 2002).

Stem Cell Potential

Stem cells with the capability of generating all tissue types of an organism, including cells of the placenta, are classified as totipotent cells. Totipotent cells include the combined sperm and egg, or germ cells. Totipotent cells give rise to pluripotent and multipotent stem cells. Pluripotent cells can differentiate into cells that make up tissues of the three germ layers: endoderm, mesoderm and ectoderm. On the other hand, multipotent cells can only differentiate into the cell types that make up the germ layer from which they came. Friedenstein demonstrated that bone marrow cells can differentiate into osteocytes of bone tissue (Friedenstein et al., 1966). However, recent reports suggest there are exceptions to these general classifications. For example, mesenchymal stem cells (MSCs) from the mesoderm have been shown to differentiate into nerve cells that are derived from the ectoderm (Pittenger et al., 1999).

Embryonic Stem Cells

Fertilization of an ovum initiates a procession of cell divisions, also known as cleavage, which drive an embryo through the various developmental stages including the zygotic stage, the morula stage and the blastula stage. The union of the haploid sperm and egg forms the diploid zygote before the onset of cell cleavage. The zygotic cell undergoes multiple cell

divisions and a process known as compaction takes place in which cells begin to stick together through cell-surface adhesion glycoprotein interactions. The inner most cells, or blastomeres, become the inner cell mass (ICM) and the outer cells form the trophoblast. At this stage, the developing embryo is referred to as a blastocyst. Blastocysts form at approximately 5 days post fertilization in the human and 7 days in the cow. Preimplantation blastocysts consist of an ICM and a fluid-filled blastocoel, which are encapsulated in a layer of cells called the trophectoderm. Cells of the ICM give rise to the three germ layers while the cells of the trophectoderm form the placental tissues.

In 1981, Evans and Kaufman and Martin and coworkers independently established *in vitro* culture of embryonic stem cells derived from the ICM of the preimplantation mouse embryo (Evans and Kaufman, 1981; Martin, 1981). Both groups utilized co-culture with fibroblast feeder layer cells that maintain embryonic stem cell pluripotency and prevent differentiation. The cells in culture demonstrate chromosomal stability (Pera et al., 2000) and differentiation potential as embryoid bodies, which recapitulate embryonic development but do not form extraembryonic tissues (Evans and Kaufman, 1981). Also, reports characterizing embryonic stem cells showed that they can integrate into the construction of all tissue types after injection into an early stage mouse embryo (Robertson et al., 1986; Thomas and Capecchi, 1987) and that they express a multitude of pluripotent stem cell surface markers (Rogers et al., 1991; Rosner et al., 1990).

Somatic Stem Cells

Human patient pathologies for progressive osseous heteroplasia (POH) and fibrosis ossificans progressiva (FOP) exhibit the formation of ectopic bone within subcutaneous adipose tissues, muscles, tendons and ligaments (Kaplan et al., 1994a; Kaplan et al., 1994b). Therefore, these afflicted tissues contain a mixture of cells, typically of mesenchymal origin, including osteoblasts (Kaplan et al., 1994a). POH can be acquired in response to burns, infections, trauma, post-surgical complications, neoplasia and neurologic disease (Puzas et al., 1989). POH is also a rare autosomal dominant inheritable disease in which a mutation in the

GNAS1 gene prevents the coupling of hormone receptors to adenylate cyclase (Shore et al., 2000). The pathologic observations associated with POH indicate the presence of progenitor cells within adult tissues.

Pathological observations such as those in POH have spurred the exploration of other sources of progenitor cells or adult stem cells. Sources of adult stem cells, also known as somatic stem cells, have been found in a multitude of tissues including bone marrow (Friedenstein et al., 1976), adipose (Rodbell, 1964; Zuk et al., 2001), cord blood (Broxmeyer et al., 1992) and skin (Toma et al., 2001). Somatic stem cells are not totipotent, but may be considered pluripotent and definitely multipotent in that they can differentiate into tissue lineages of the germ layer from which they came. At this time, hematopoietic stem cells (HSCs) are the best characterized of somatic stem cells and multiple studies have been performed to illustrate their plasticity or ability to give rise to progeny of different cell types. Jahagirdar and Verfaillie demonstrated that not only could HSCs give rise to fat, bone, muscle and cartilage but also to gut, liver, lung and endothelial cells (Jahagirdar and Verfaillie, 2005). Also in 2005, Mezey demonstrated that HSCs could differentiate into brain cells (Mezey, 2005). These reports along with many others support the theory that somatic stem cells possess a more pluripotent capacity rather than the stricter multipotent characteristic as once thought.

Embryonic Stem Cells vs. Somatic Stem Cells

Under optimal *in vitro* culture conditions, somatic stem cells can demonstrate expansion rates of up to approximately 50 population doublings (PDs) (Friedenstein et al., 1974) similar to all other eukaryotic cell lines cultured *in vitro* as defined by the Hayflick limit (Hayflick and Moorhead, 1961). However, embryonic stem cell populations are believed to possess a longer lifespan *in vitro*. Human embryonic stem cells have maintained an undifferentiated state and a normal karyotype *in vitro* for up to 250 PDs (Amit et al., 2000). These observations suggest that embryonic stem cells may in fact be more useful in their applications than somatic stem cells.

The longer lifespan of embryonic stem cells compared with somatic stem cells may be attributed to the length of telomeric DNA and telomerase activity. In 1985, Carol Greider and Elizabeth Blackburn discovered the enzyme telomerase in the ciliate *Tetrahymena* (Greider and Blackburn, 1985). Telomerase is responsible for adding TTAGGG specific sequence repeats to the 3' end of DNA following each replication cycle (Greider and Blackburn, 1985). The condensed repetitive DNA sequence, also known as the telomere, protects the chromosome ends from homologous recombination and non-homologous end joining. The present understanding is that a decline in telomerase activity is associated with aging cells and that telomere length shortens as a result of incomplete replication (Counter et al., 1992; Harley et al., 1992). As telomere length shortens, the cell progresses closer to its replicative limit, accommodating Leonard Hayflick's postulation of limited somatic cell division (Hayflick and Moorhead, 1961), therefore advancing into replicative cellular senescence (Counter et al., 1992; Harley et al., 1992). Embryonic stem cells and malignant cells have been shown to have elevated levels of telomerase activity (Kim et al., 1994) lending to the virtual immortality of embryonic stem cells *in vitro* and the evasion of cancer cells from programmed destruction. However, somatic stem cells and somatic cells in general possess low levels of telomerase activity if any at all (Rubin, 2002; Wright and Shay, 2002).

Although embryonic stem cells may prove to be more versatile in therapeutic applications, much controversy surrounds the use of stem cell sources derived from human embryos and fetuses for biomedical treatments. The current media has illuminated stem cell research in a negative light by portraying all stem cells as those only from embryonic origin at the price of human embryo destruction. Also, the inability to completely purify embryonic stem cell populations for use in clinical applications has maintained the potential for cancerous tumor formation. The public belief has influenced the political circuit and as a result, the U. S. National Institutes of Health lists only 21 embryonic stem cell lines available for distribution for research nationwide in 2009.

Therefore, the controversy has spurred research of alternative stem cell sources. The use of adult or somatic stem cells, found in adult tissues, escapes the controversy and also eludes immunological rejection and disease contraction (Merok and Sherley, 2001). Diligent efforts of current research are now focusing on the isolation and characterization of adult or somatic stem cells. Somatic stem cells can be found among differentiated tissues, however current research is focusing on more easily attainable sources with less patient discomfort. Ongoing research is also concentrating on the isolation and culture of large numbers of cells that will uniformly differentiate into a functional state to then become eligible for applications in cell therapies (Tsai et al., 2002).

Mesenchymal Stem Cells

To this date, many scientists are working vigorously to ascertain the true stem cell renewal capabilities of those cells referred to as mesenchymal stem cells or stromal cells or MSCs. It remains to be determined whether they can demonstrate the ability to divide producing a daughter cell that remains a precursor cell as well as produce a daughter cell that will differentiate along a specific mesenchymal cell lineage. For the purpose of terminology, MSCs are a type of somatic stem cell found in adult tissues such as adipose tissue, bone marrow and cord blood that are believed to be, for the most part, pluripotent precursors. In 1970, Friedenstein and colleagues first identified MSCs isolated from bone marrow as fibroblast precursors and referred to them as colony-forming unit-fibroblastic (CFU-f), which describes fibroblastic colony growth (Friedenstein et al., 1970). Traditionally, MSCs have been isolated from various tissues based on their ability to adhere to plastic and their morphology similar to that of fibroblasts (Kassem et al., 1993; Luria et al., 1971; Rickard et al., 1996). Depending on the culture conditions, MSCs can be cultured *in vitro* as pluripotent cells or as differentiated cells. MSCs support differentiation into adipocyte, osteoblast and other mesodermal cell types (Gimble et al., 1990; Gimble et al., 1996; Nuttall and Gimble, 2000; Owen, 1988).

As previously stated, HSCs have been more extensively characterized than any other somatic stem cell type. However, the niche or environment in which the HSC develops and functions is still very poorly understood. Recent studies have revealed that cells derived from MSCs, such as osteoblasts and fibroblasts, interact directly with functioning HSCs. MSCs have also been shown to play major roles in the regulation of hematopoiesis (Mitsiadis et al., 2007). Molecules produced by MSCs have a potent paracrine effect within the niche that ultimately regulates the proliferation rate of HSCs and their function (Orkin and Zon, 2008; Wilson and Trumpp, 2006).

A multitude of reports describe the cell surface phenotypic profile of MSCs. However, through the extensive exploration, conflicts in results have prevented the establishment of inimitable, identifiable cell markers for MSCs. Haynesworth and coworkers demonstrated that SH2, SH3 and SH4 can be used to distinguish MSCs from contaminating HSC populations (Haynesworth et al., 1992). In addition, STRO-1 antibodies have been employed to select for MSCs in bone marrow by magnetic activated cell sorting (Dennis et al., 2002; Gronthos et al., 1994; Tamayo et al., 1994). STRO-1 selected cells have been shown to be chondrogenic, adipogenic, and support hematopoiesis (Dennis et al., 2002) as well as osteogenic (Gronthos et al., 1994). Gene and protein expression profiles in some species specific MSCs show the presence of cell surface markers, such as CD34, CD44 (Gronthos et al., 2001) and SSEA-4 (Gang et al., 2007). Yet, according to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, a cell can only be classified a MSC if proved to be negative for CD11b, CD14, CD19, CD34, CD45, CD79a and HLA-DR and positive for CD73, CD90 and CD105 (Dominici et al., 2006).

MSCs in culture exhibit a high degree of plasticity as suggested above. The process of differentiation was traditionally thought to be an irreversible pathway to a committed cell lineage. However, differentiated MSCs possess the ability to dedifferentiate and transdifferentiate into other cells of mesenchymal origin such as adipocytes, chondrocytes and osteoblasts (Song and

Tuan, 2004). Song and Tuan even rule out the potential for progenitor cell contamination within the originating stem cell pool and cell-to-cell fusion of progenitor cells and tissue-specific cells (Song and Tuan, 2004). In addition, human articular chondrocytes have been shown to differentiate into osteogenic and adipogenic pathways and cultured adipocytes into the chondrogenic or osteogenic pathways (Sato et al., 2005; Tallheden et al., 2003). In spite of these findings, research continues to explore the specific dedifferentiation and transdifferentiation capabilities of MSCs.

MSCs have been applied in cell-based therapies for the treatment of such conditions as bone and cartilage defects, injuries to spinal cord and nervous system, lung diseases and cardiac disorders. As mentioned above, MSCs exert paracrine effects by the secretion of bioactive molecules making them appealing for use in cell-based therapies. MSCs have been injected following bone marrow transplants in order to speed up patient recovery (Le Blanc et al., 2004). Also, tissue engineering using the combination of MSCs and scaffolds of a specified biomaterial and shape can be used in grafts (Kojima et al., 2004) with minimal host rejection (Le Blanc et al., 2004).

MSC Cell Surface Phenotype

In the field of stem cell biology it is important to be able to recognize, identify and distinguish one specific stem cell population from another. Multiple techniques exist for the isolation of plastic-adherent multipotent cells from adult tissue sources, however, all render a heterogeneous population of cells with discrepant proliferation characteristics and differentiation potentials. Identifiable stem cell populations are necessary in order for researchers to be able to describe localization, function and therefore mechanism of tissue repair. The quest for a cell-surface antigen profile for MSCs of all types is presently ongoing. Consequently, the understanding as to what a MSC actually is requires better definition.

The current method of physical phenotyping of MSCs populations relies on a combination of cell surface markers briefly described above. There is a plethora of reported

positive cell surface markers for MSC identification in which different laboratories employ different combinations of markers. Consequently, it is important to keep in mind that the lack of unique, standardized molecules leaves considerable room for generating discrepant results from varying research groups. As mentioned above, SH2, SH3, SH4 and STRO-1 are among the most well known MSC surface markers. SH3 and SH4 are monoclonal antibodies that were raised against CD73, a 5' ecto nucleosidase, and were developed with specificity for MSCs (Haynesworth et al., 1992). The specificity for CD73 of MSCs prevents the selection of HSCs, osteoblasts and other contaminating cell populations. There are, however, discrepancies to consider when relying on STRO-1 alone in general MSC identification. STRO-1 expression is unfortunately not limited to MSCs, and unlike CD73, STRO-1 expression is lost through expansion in culture (Gronthos et al., 2003). The decline in STRO-1 expression restricts the use of STRO-1 as an identifiable marker to primary and early MSC cultures only (Gronthos et al., 2003). It has been proposed that the use of STRO-1 is better exploited in combination with other MSC identifying cell surface markers (Gronthos et al., 2003).

Vascular cell adhesion molecule-1 or CD106 has been identified as a cell surface marker that can be used in conjunction with other markers for positive MSC population identification. Considering the caveats of utilizing STRO-1 alone, Gronthos and coworkers demonstrated that CD106 amplified the selection of multipotential, colony forming cells that coexpress STRO-1 with high telomerase activities (Gronthos et al., 2003). Other positive MSC cell surface markers include CD10 (metalloproteinase; CALLA), CD13 (metalloproteinase; Aminopeptidase N), CD14, CD29 (integrin β 1; fibronectin receptor), CD44 (adhesion receptor molecule; hyaluronate), CD49a, CD63, (HOP-26), CD71, CD73, CD90 (Thy-1), CD105 (adhesion receptor molecule; Endoglin), CD120a, CD124, CD166 (SB-10), CD271 (NGFR), CD309 (Flk-1) and stem cell antigen-1 (Sca-1) (Gronthos et al., 1994; Koc et al., 2000; Mitchell et al., 2006; Pittenger et al., 1999; Stewart et al., 2003). However, it is important to note that

other researchers have reported conflicting results concerning MSC expression of CD10, CD90, CD309, Sca-1 and others.

Selection of a homogeneous population of MSCs might be accomplished with the use of both positive and negative cell surface markers. Markers that have been reported not to be present upon the MSC surface include: CD11b, CD14, CD19, CD31, CD34, CD45, CD79a, CD117 and HLA-DR (Colter et al., 2001; da Silva Meirelles et al., 2006; Pittenger et al., 1999).

MSC Self-renewal and Maintenance

A recent shift in MSC research has focused on unraveling the intricate machinery involved in stem cell self-renewal and maintenance. The mechanisms of self-renewal function to preserve the undifferentiated state of the stem cell and are dictated by extrinsic signals and several intrinsic factors including epigenetic modifications and small RNA regulators. Leukemia inhibitory factor (LIF), multiple growth factors and other soluble factors such as Wnt, Notch and Sonic hedgehog (Shh) have all been implicated in the mediation of stem cell self-renewal and maintenance. The detailed mechanism of the LIF pathway is unknown, however it has been shown to maintain the undifferentiated state of MSCs (Jiang et al., 2002) as well as activate/repress bone cell activities (Heymann and Rousselle, 2000).

Growth factors such as epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2) and transforming growth factor-alpha (TGF- α) regulate stem cell replication via the extracellular environment (Reynolds et al., 1992; Reynolds and Weiss, 1992; Tsutsumi et al., 2001; Zaragosi et al., 2006). Apparently, EGF, FGF-2 and TGF- α play a role in the control of receptor tyrosine kinase signaling essentially preserving the stem state of MSCs and extending their viability in culture (Tsutsumi et al., 2001; Zaragosi et al., 2006).

Wnts have been shown to be major players in the proliferation of adult human MSCs by initiating the cascade involving β -catenin and cyclin D1 (Boland et al., 2004; Willert and Nusse, 1998). Wnt3a specifically increased MSC proliferation while suppressing osteogenic differentiation (Boland et al., 2004). Notch functions as a paracrine factor released from a

neighboring cell within the MSC niche, which transduces signals into the nucleus of the cell affecting gene expression for maintaining the undifferentiated state (Jarriault et al., 1995; Yoon and Gaiano, 2005). Lastly, Shh is also considered an important component of enhancing proliferation capacity of MSCs. Hepatic stellate cells increased Shh expression after coculture with MSCs, in which stellate cells then served in a paracrine fashion to elevate the proliferation and differentiation of the MSCs delivered through treatment of liver fibrosis (Lin et al., 2008).

Stem cell self-renewal and the maintenance of the undifferentiated state are also controlled by epigenetic modifications. These modifications include histone acetylation, methylation, phosphorylation, ubiquitination, sumoylation, citrullination and ADP-ribosylation (Strahl and Allis, 2000). The expression levels of the enzymes responsible for producing these modifications ordain epigenetic control. For example, DNA methyltransferases (Dnmt) add covalent methyl groups ($-\text{CH}_3$) to cytosine bases located within the promoter region of a gene (Geiman and Robertson, 2002). The resultant methyl group is associated with transcriptional silencing as a result of the inability of transcription factors to bind to methylated DNA sequences (Hiendleder et al., 2004a). Also, histone acetylation occurs through the histone acetylases or HATs and modifications are removed via histone deacetylases or HDACs. The removal of histone acetylated groups primarily from histone H3 and H4 causes significant changes in the shape or configuration of the chromatin that then prevents the access of transcription factors to bind DNA sequences (Cezar, 2003). Without access to the DNA, transcription cannot commence, therefore the cell remains in a stem state all on account of the specific interactions between the DNA and chromatin remodeling proteins.

The epigenetic landscape created by these modifying enzymes has been under close investigation recently. Methylation modifications have been described on targeted genes just downstream to a variety of developmentally important transcription factors such as Oct4, Nanog, Sox2, c-Myc, and Klf4. These particular transcription factors have been implicated in human development and are believed to be necessary for the maintenance of pluripotent and

the regulation of self-renewal pathways (Boyer et al., 2005). Stem cell self-renewal under transcriptional control is associated with the degree of lysine methylation including mono-, di- or tri-methylated histones. Dnmts and demethylases facilitate chromatin modifications to form a described “bivalent” conformation that consist of regions of histone 3 (H3) lysine 27 methylation harboring smaller regions of H3 lysine 4 methylation (Bernstein et al., 2006). Studies show that methylation of lysine 4 positively mediates transcription by recruiting histone acetylases and other chromatin remodeling enzymes (Santos-Rosa et al., 2003) while lysine 27 negatively mediates transcription by preserving a closed chromatin conformation (Francis et al., 2004; Ringrose et al., 2004). The methylation pattern functions to repress transcription while holding the chromatin in a potentially activate state. Other transcriptionally repressive demarcations include H3 trimethyl lysine 9, H3 monomethyl lysine 20 and H3 lysine 27 methylation, and all can be found in neuronal stem cells (Shi et al., 2008; Wernig et al., 2007).

Small non-coding RNA regulators also control cell proliferation and other cellular processes involved in development (Wienholds et al., 2005). Micro-RNAs (miRNA) are single-stranded RNA fragments transcribed from DNA but not translated into proteins. Primary miRNA transcripts (pri-miRNA) are processed within the nucleus into short stem-loop or hairpin-like structures called pre-miRNAs. These pre-miRNAs translocate into the cytoplasm where they interact with the endonuclease Dicer that cleaves pre-miRNA into two complementary RNA molecules. One of the complements created integrates into a RNA-induced silencing complex or RISC and guides the complex to complementary portions of messenger RNA. The interaction represses translation thereby preventing differentiation either by a physical block or the cleavage of target mRNAs.

MSC Differentiation

Following the successful isolation and identification of a cell population thought to be MSCs, further characterization must be performed to determine the ability of the population to differentiate. The elucidation of specific signaling networks involved in the differentiation of

MCSs remains unclear. However, several intrinsic as well as extrinsic factors from the cell's environment, as mentioned above, influence cell and tissue differentiation.

MSCs exist in their respective niche within varying tissues as quiescent cells arrested in the G0/G1 cell cycle stage until an extracellular cue from the cell's environment induces proliferation and differentiation of that cell (Baksh et al., 2004). MSCs are adult or somatic stem cells and, also as mentioned above, divide in an asymmetric manner where the tissue progenitor cells give rise to two daughter cells, one that remains a progenitor cell and one precursor cell that begins the process of terminal differentiation. The resultant progenitor cell goes on to continue the asymmetric division pattern to replenish the MSC population within the tissue from which it came for future use in tissue repair or regeneration in the case of injury or disease. The precursor cell formed can further divide to produce multipotent cells that can differentiate along specific cell lineages such as chondrocytes, osteoblasts, adipocytes and myocytes. Cytokines, extracellular matrix proteins and other soluble factors are responsible for dictating this division process and the timing of their function is likely very important (Baksh et al., 2004).

In order to facilitate the development of optimal culture conditions of MSCs for appropriate clinical applications, Baksh and coworkers investigated the presence of a myriad of transcripts before and after the induction of lineage differentiation including osteogenesis, chondrogenesis and adipogenesis. They propose a model for the regulation of MSC differentiation suggesting that due to the presence of many common genes, osteogenesis, chondrogenesis and adipogenesis are likely governed by a single master control mechanism (Baksh et al., 2004).

MSC Differentiation: Chondrogenesis

Molecular investigations have identified multiple instructive signaling molecules that induce the expression of specific chondrogenic genes. The discovery of these molecules has led to their incorporation into culture medium allowing chondrogenic differentiation *in vitro*.

Kisiday et al. compared bone marrow and adipose tissue-derived MSCs for their ability to express benchmark chondrogenic genes after culture in media with and without transforming growth factor- β 1 (TGF- β 1) and found that TGF- β 1 media did in fact upregulate the expression of collagen type II (Kisiday JD, 2007). MSC-derived chondrocytes then positively express chondrogenic-associated markers including transcription factor Sox-9 (Sry-type high mobility group box containing gene 9) and extracellular matrix genes such as collagen II, collagen IX, aggrecan, decorin, glycan and cartilage oligomeric matrix protein (Baksh et al., 2004; Tuan et al., 2003). Additional research indicates that chondrogenesis of MSCs can be induced by the presence and combination of other molecules including TGF- β 3, bone morphogenetic protein-2 (BMP-2), BMP-4, BMP-6 (Boskey et al., 2002; Gooch et al., 2002), BMP-12 (Gooch et al., 2002), BMP-13 (Gooch et al., 2002; Nochi et al., 2004), growth and differentiation factor-5 (GDF-5) (Tuan et al., 2003), ascorbic acid and dexamethasone (Awad et al., 2003; Erickson et al., 2002; Guilak et al., 2004; Zuk et al., 2001).

Ongoing investigations are exploring the roles of TGF- β s and BMPs in MSC differentiation mechanism because they signal through mitogen-activated protein kinases (MAPKs) and R-Smad proteins. The MAPK cascades phosphorylate HATs that then bind R-Smad proteins (Abecassis et al., 2004). Again, HATs modulate the addition of an acetyl group to histones thereby opening up chromatin conformation increasing the accessibility of transcription factors to bind DNA sequences and inducing chondrogenesis in MSCs.

MSC Differentiation: Osteogenesis

Recent data suggests that there is most likely a close relationship between the processes of adipogenesis, osteogenesis and chondrogenesis in MSCs supporting the presence of multiple common genes observed by Baksh et al. (Baksh et al., 2004). However, it is proposed that adipogenesis and osteogenesis have more regulatory control genes and precursors in common than those involved in the chondrogenesis pathway (Baksh et al., 2004; Muraglia et al., 2000). Regulating transcription factors have a completely opposite effect on the

control of osteogenesis (Beresford et al., 1992; Nuttall and Gimble, 2004). The inverse communication between these committed cell lineages instigates the potential for exploitation of the phenomenon in therapeutic applications.

Peroxisome proliferator-activated receptor γ (PPAR γ) is an important regulator of both adipogenesis and osteogenesis. While PPAR γ promotes adipogenesis of MSCs it sequesters osteogenesis (Nuttall and Gimble, 2004). Runx2 is a major regulating gene for the onset of osteogenesis. Runx2 interferes with cyclin-dependant kinase inhibitors p21 and p27 lifting cell cycle arrest and allowing the cell to progress into the S phase (Lian et al., 2006). PPAR γ blocks DNA binding of transcription factor Runx2, therefore preserving the quiescent state of the cell and blocking the development of the osteogenic phenotype (Lian et al., 2006). Also, histone deacetylases 4 and 5 remove acetyl groups from Runx2 allowing for its degradation by Smurf1, Smurf2 and E3 ubiquitin ligases (Jeon et al., 2006). Tumor necrosis factor- α (TNF- α), a cytokine involved in systemic inflammation, also induces Runx2 degradation by stimulating increased expression of Smurf1 and Smurf2 (Kaneki et al., 2006). On the other hand, BMP-2 modulates the action of Runx2 by inducing p300 recruitment of histone acetylases, which in turn enhance osteogenic differentiation (Jeon et al., 2006).

MSC Differentiation: Adipogenesis

PPAR- γ can be inhibited by many factors including those described above as well as by a variety of cytokines and growth factors. Regardless, PPAR- γ is the central regulatory unit for the promotion of MSC adipogenesis. PPAR- γ complexes with the retinoid X receptor (RXR) that together causes the activation of CCAAT/enhancer binding proteins (C/EBP) (Rosen and Spiegelman, 2000). C/EBPs play a major role in the maturation of adipose tissue and without their expression adipogenesis is severely retarded as demonstrated in knockout mice (Tanaka et al., 1997).

Common components of adipogenic-inducing culture media include: methylisobutylxanthine, dexamethasone, insulin and fetal bovine serum, which by some unknown mechanism induce the action of C/EBP family members. Specifically, C/EBP α activates multiple genes unique to mature differentiated adipocytes including stearoyl CoA desaturase (SCD1), insulin-responsive glucose transporter-4 (GLUT-4), phosphoenolpyruvate carboxykinase (PEPCK) and UCP genes that have functional binding sites within their promoter regions (Christy et al., 1989; Park et al., 1990; Yubero et al., 1994).

The toggle between stretch and non-stretch induced pathways dictates tension-induced/inhibited protein (TIP) activation. TIPs are chromatin remodeling proteins with inherent HAT activity. The mechanical force of tension or “stretch” inflicted upon the MSCs will affect the lineage of differentiation that the cell will pursue (Jakkaraju et al., 2005). Under non-stretch conditions TIP-1 is activated and promotes the onset of adipogenesis (Jakkaraju et al., 2005). On the other hand, static stretch will activate the TIP-3 pathway leading to the induction of myogenesis (Jakkaraju et al., 2005). On some occasions non-stretch conditions will induce TIP-1 activation through RhoA mediation (McBeath et al., 2004). Through this unique mechanism, cell shape alters the cytoskeletal structure, which in turn fires signals altering transcription and therefore differentiation potential of the MSC.

MSC Differentiation: Myogenesis and Tenogenesis

The induction of myogenesis for muscle generation and tenogenesis for tendon generation has also been studied. Bone marrow MSCs have been shown to differentiate into muscle tissue for treatment following muscle degeneration when transfected with an active form of the single-pass transmembrane receptor Notch-1 (Dezawa et al., 2005). Further research has identified the ligand for the Notch-1 transmembrane receptor as Jagged 1, also known as CD339 (Li et al., 2006). Li and coworkers cocultured MSCs with cardiomyocytes from neonatal rat ventricles producing an inductive environment for the differentiation of MSCs into cardiomyocytes (Li et al., 2006). Soluble Jagged 1 was added to the culture media, which

enhanced myogenesis of the MSCs through the Notch-1 pathway (Li et al., 2006). A mechanism involving R-Smad8 and BMP-2 has been shown to cause differentiation of MSCs into tenocytes (Hoffmann et al., 2006). An active form of Smad8 was cotransfected with BMP-2 into MSCs, which produced morphological changes and gene expression profiles similar to tenocytes (Hoffmann et al., 2006). *In vitro* culture of induced myogenic and tenogenic cell populations will be very useful in the treatment of these tissues when damaged or diseased.

Adipose Tissue

Adipose tissue can be found throughout the entire human body. There are two different types of adipose tissue that are physiologically unique from each other and have completely different functions: white adipose tissue (WAT) and brown adipose tissue (BAT). The cells comprising WAT often contain one large lipid filled vacuole that takes up the majority of the cell itself leaving just enough room for the nucleus. WAT can be found subcutaneously and in visceral depots. It functions primarily as an energy reservoir but also plays roles in lipid metabolism, insulation, mechanical cushioning, structural support, and regulating hematopoiesis and osteogenesis. BAT cells contain many lipid filled vacuoles and multiple mitochondria that give the cells their brown color (Hull and Segall, 1966). BAT is located in the interscapular region and around major organs such as the heart and lungs. Energy is converted into heat within these cells to maintain body heat in early youth and hibernating animals.

Over the past several decades, the prevalence of overweight and obese people has drastically increased. The worldwide epidemic has drawn the attention of many research arenas. A Pubmed search for “obesity” over the past 60 years brings up articles that have increased progressively with each decade. With the infiltration of technology in our everyday lives and an abundant food supply, obesity in the United States has reached astronomical proportions and has become the number one concern in childhood health.

Obesity results when a severe imbalance between energy intake and expenditure occurs. Adipose tissue by nature serves as a reservoir for energy to be used during periods of

starvation. As long as energy intake exceeds expenditure, adipocytes will accommodate the storage of energy by increasing in size and number until the balance is tipped and energy expenditure begins to deplete the supply. However, in 1999, Roger Cone proposed a model for the regulation and maintenance of a certain volume of adipose tissue within an individual. A drop in the overall volume of adipose tissue due to dieting, strenuous exercise or liposuction triggers a homeostasis modulator to return the total adipose tissue volume to its original level. Also, classic experiments performed in rats by Faust et al. demonstrate that the removal of inguinal fat pads stimulated regeneration within 7 months of surgery (Faust et al., 1977). Therefore, the processes involved in adipogenesis leading to obesity suggests the presence of a population of stem cells located in specific fat depots.

Adipose-derived Stem Cells

MSCs have been isolated from a myriad of mammalian tissues including the placenta (Yen et al., 2005), nervous tissue (Baksh et al., 2004), brain, thymus, lung, kidney, liver, spleen (da Silva Meirelles et al., 2006), brain (Jiang et al., 2002) and pancreas (Hu et al., 2003). However, MSCs from bone marrow have been more thoroughly characterized than any other type of MSC most likely due to accessibility. Obtaining this tissue requires a relatively uncomplicated yet painful procedure. Adipose tissue, on the other hand, represents a tissue source that is extremely abundant (*i.e.*, via discarded lipoaspirate tissue), readily accessible, results in minimal patient discomfort and yields high enough cell numbers to sufficiently and efficiently expand cell populations.

Isolation Procedures

Martin Rodbell is accredited with the first description for the isolation of adipose tissue from rats in 1964 (Rodbell, 1964). Briefly, the procedure involved the removal of tissue, fine mincing and exposure to, at the time, a commercially available crude preparation of collagenase (Type I). The digested tissue was then subjected to multiple centrifugation steps yielding the separation of floating cells, which were adipocytes, from the pelleted, heterogenous stromal-

vascular fraction (Rodbell, 1964). Several research groups since then, including Van and Roncari, Deslex et al. and Hauner et al., have shown that the isolated fraction of cells contains plastic-adhering adipocyte precursors (Deslex et al., 1987; Hauner et al., 1989; Van and Roncari, 1977). Modified versions of this protocol have been adapted for the isolation of adipocyte precursors or adipose-derived stem cells (ASCs) from the rat, mouse, pig, horse and human. Human adipose tissue has been obtained through processed liposuction aspirates, which are discarded in a typical procedure (Deslex et al., 1987; Hauner et al., 1989; Lalikos et al., 1997; Moore et al., 1995; Van and Roncari, 1977). Obtaining ASCs from liposuction aspirates is beneficial in that it bypasses several steps in the original procedure.

ASC Characterization: Immunophenotype

Due to the easy access to human adipose tissue described above, much of the research investigating the immunophenotype of ASCs has been performed in the human. Previously stated, MSCs are positive for multiple cluster differentiation molecules such as CD73, CD105 and CD90 and negative for CD11b, CD14, CD45 and HLA-DR. The presence, or absence, of these markers was consistent for the human ASC cell surface. Other positive antigens include CD9, CD29, CD44, CD49, CD166, osteopontin, osteonectin, vimentin, HLA-ABC and various others (Table 2.1) (Aust et al., 2004; Gimble et al., 2007; Gronthos et al., 2001; McIntosh et al., 2006; Mitchell et al., 2006; Zuk et al., 2002). Human ASCs produced additional negative results for CD16, CD18, CD31, CD50, CD56, CD62 and CD104 (Table 1)(Aust et al., 2004; Gimble et al., 2007; Gronthos et al., 2001; McIntosh et al., 2006; Mitchell et al., 2006; Zuk et al., 2002).

ASC Characterization: Differentiation

In addition to the determination of the phenotypic landscape, the differentiation capacity must be evaluated to further characterize ASCs. Vidal et al. (2007) determined the multipotentiality of equine ASCs. Following adipogenesis induction, accumulation of oil droplets by Oil-red-O staining along the periphery of preadipocytes was noted followed by the formation of fat globules by day 2 following induction (Vidal et al., 2007). In addition to adipogenesis, the

Table 2.1. Immunophenotype of Passaged Human ASCs

Antigen Category	Surface-Positive Antigens	Surface-Negative Antigens
Adhesion molecules	CD9 (tetraspan), CD29 (β_1 integrin), CD49 days (α_4 integrin), CD54 (ICAM-1), CD105 (endoglin, CD166 (ALCAM)	CD11b (α_b integrin), CD18 (β_2 integrin), CD50 (ICAM-3), CD56 (NCAM), CD62 (E-selectin), CD104 (α_4 integrin)
Receptor molecules	CD44 (hyaluronate), CD71 (transferrin)	CD16 (Fc receptor)
Enzymes	CD10 (common acute lymphocytic leukemia antigen), CD13 (aminopeptidase), CD73 (5' ecto-nucleotidase), aldehyde dehydrogenase	
Extracellular Matrix molecules	CD90 (Thy-1), CD146 (Muc18), collagen types I and III, osteopontin, osteonectin	
Cytoskeleton	α -smooth muscle actin, vimentin	
Hematopoietic		CD14, CD31, CD45
Complement cascade	CD55 (decay-accelerating factor), CD59 (protectin)	
Histocompatibility	HLA-ABC	HLA-DR
Antigen		
Stem Cell	CD34, ABCG2	
Stromal	CD29, CD44, CD73, CD90, CD166	

(Gimble et al., 2007)

osteogenic potential was demonstrated in these equine ASCs by the presence of calcium mineralization through alizarin red positive calcium phosphate nodule formation (Vidal et al., 2007). Human ASCs have been more extensively characterized in terms of differentiation potential and have been shown to successfully respond to chondrogenic, osteogenic, myogenic, adipogenic and neurogenic conditions. The presence of TGF- β , ascorbate and dexamethasone in the ASC culture environment will induce the expression of chondrogenic proteins including collagen Type II, Type IV and aggrecan (Awad et al., 2003; Erickson et al., 2002; Wickham et al., 2003). Although culture components are similar to those in chondrogenic inducing conditions, osteogenesis marked by calcium phosphate mineralization within the extracellular matrix will ensue with the inclusion of ascorbate, dexamethasone, in addition to β -glycerophosphate (Halvorsen et al., 2000; Halvorsen et al., 2001b). Finally, an induction cocktail consisting of insulin, methylisobutylxanthine, dexamethasone and indomethacin will cause ASCs to form lipid-filled vacuoles that secrete leptin and lipoprotein lipase (Halvorsen et al., 2001a; Hauner et al., 1989; Zuk et al., 2001).

ASCs as Donor Cells for Nuclear Transfer

Currently, nuclear transfer results in relatively low success rates and research has reported substantial problems with the welfare of some of the cloned animals produced. Developmental abnormalities observed following nuclear transfer include low pregnancy rates, high percentages of loss during early and late pregnancy, stillbirths, early postnatal deaths, short life-span, obesity and malformations. These abnormalities make up what is referred to as Large Offspring Syndrome or LOS and may be caused by several factors, such as using an inappropriate donor cell and/or recipient oocyte, the inability to properly synchronize the cell cycle phase of the donor nucleus and the recipient cytoplasm, inadequate reprogramming of the donor genome, inappropriate handling of oocytes, somatic cells and embryos during maturation, and/or subsection of these cells to the various necessary manipulations and cultural techniques which could result in cellular stresses (Young et al., 2001).

Epigenetic Modifications and Nuclear Transfer

Much research has focused on the epigenetic modifications of DNA and their role in embryonic development. In the production of a normal developing embryo, the oocyte (arrested at metaphase II) is united with a sperm where it completes meiosis and extrudes the second polar body. The embryo contains a subset of cells that remain totipotent (can support differentiation into all cell types) for the first one to two cell divisions (Kono, 1997). The following cleavage events undergo DNA modifications, which render them unable to form all tissue types and once initiated the series of events proceeds beyond the point of no return. However, exposure of a donor cell's chromatin to ooplasm as in nuclear transfer will redirect the programming of the donor nucleus to mimic that of a zygotic nucleus as in normal fertilization (Figure 2.1) (Kikyo and Wolffe, 2000). The oocyte virtually erases the epigenetic modifications imposed on the genome during differentiation and reinstates totipotency (Kikyo and Wolffe, 2000). The reprogramming of the donor cell nucleus is essential to initiate and complete embryonic development.

Fusion with embryonic stem cells provides an alternative method for inducing nuclear reprogramming of somatic nuclei. Factors within the embryonic stem cell, much like in the unfertilized oocyte, render a resultant hybrid cell, which demonstrates pluripotent competence by differentiation into multiple cell lineages and expression of tissue-specific genes (Cowan et al., 2005; Tada et al., 2001). Several somatic cell types have been successfully reprogrammed upon fusion with embryonic stem cells including thymocytes (Tada et al., 2001), human fibroblasts (Cowan et al., 2005) and human myeloid precursor cells (Yu et al., 2006). Research indicates that the nuclear compartment of the embryonic stem cell houses the reprogramming capability because fusion of karyoplasts and not cytoplasts generate the reprogrammed hybrid (Do and Scholer, 2004). These reprogrammable epigenetics govern the regulation of repression of genes not necessary in cell types at specific developmental stages while preserving the DNA

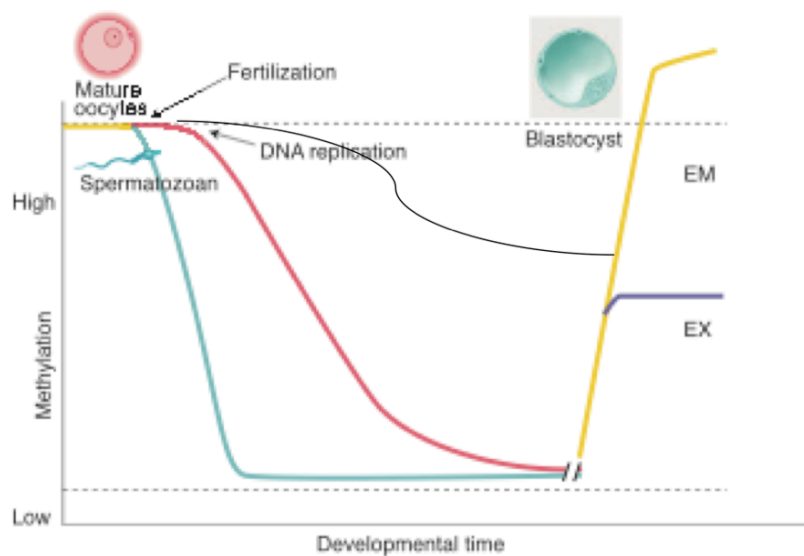


Figure 2.1 Epigenetic reprogramming of methylation/demethylation patterns in preimplantation embryos. The paternal genome (blue line) is demethylated immediately following fertilization via active demethylation whereas the maternal genome (red line) is demethylated through passive mechanisms shortly after. The event of demethylation in nuclear transfer embryos (black line) is not believed to undergo complete erasure compared to the levels achieved by maternal and paternal genomes (Modified from Reik et al., 2001).

sequence (Wolffe and Matzke, 1999). Epigenetic modifications during the reprogramming process following nuclear transfer include DNA methylation and posttranslational acetylation, phosphorylation, and methylation of histone amino termini that function to modulate chromatin structure, which ultimately regulates transcriptional activation or repression of specific genes. Acetyltable lysine residues in the N termini of histone H3 (such as K9, K14, K18, and K23) and H4 (such as K5, K8, K12, and K16) and methyltable lysine residues in H3 (such as K4, K9, K27, K36, and K79) and H4 (K20) are the most relevant that have been identified (Kimura et al., 2004). Histone acetyltransferases (HATs) are the enzymes responsible for the transfer of acetyl groups to lysine residues and histone deacetylases (HDACs) dictate their removal. Acetylation neutralizes the positive amino acid charge decreasing the affinity for DNA. The termini displace from the nucleosome increasing accessibility of transcription factors, which leads to gene expression (Grunstein, 1997).

Alternatively, cytosine methylation demarcates or “imprints” portions of DNA in order to distinguish between silenced and expressed genes of parental alleles (Cezar, 2003). Generally, DNA methylation blocks transcription factors from binding methylated sequences and is associated with transcriptional silencing and imprinting (Hiendleder et al., 2004b). For example, H19, insulin-like growth factor II, and the insulin-like growth factor II receptor all display alternative DNA methylation patterns based on the inheritance of the allele either from the sperm or the oocyte (Kono, 1997). In nuclear transfer, genes normally expressed during embryogenesis are silent in somatic donor cells and must be turned back on. Therefore, the DNA methylation patterns must be reorganized to resemble that of undifferentiated nuclei (Rideout et al., 2001) (Figure 2.1). LOS and other phenotypic abnormalities may be a result of improper expression of imprinted genes as a result of reorganized DNA methylation (Kono, 1997).

Epigenetic Regulation of Pluripotency

The initial epigenetic landscape of the DNA sequence determines the pluripotent capabilities of the cell. Embryonic stem cells possess transcriptionally active chromatin that is structured in an open configuration containing an abundance of acetylated lysine residues (Boyer et al., 2006). However, as the cell commits to a differentiation pathway, acetylated histone modifications decrease, heterochromatin formation ensues, and pluripotential declines (Meshorer and Misteli, 2006; Meshorer et al., 2006).

Regulators of development in undifferentiated cells are suggested to be silenced by the presence of a bivalent domain modification pattern in which a large region of histone H3 lysine 27 trimethylation (H3K27me3) harbors smaller regions of histone 3 lysine 4 trimethylation (H3K4me3) (Bernstein et al., 2006). Important to the maintenance and pluripotency of stem cells Polycomb-group proteins catalyze the H3K27me3 modification and Trithorax group proteins catalyze the H3K4me3 modification (Bernstein et al., 2006). The dual marks contain both repressive (H3K27me3) and activating (H3K4me3) modifications, which work to silence developmental genes in embryonic stem cells while simultaneously keeping them poised for activation. As the cell differentiates, the bivalent conformation resolves where H3K4me3 activating marks permit early-replicating gene expression in lineage-committed cells and H3K27me3 repressing marks block late-replicating gene expression in lineage-committed cells (Boyer et al., 2006; Meshorer and Misteli, 2006).

Also of note, these developmental regulators contain highly conserved non-coding elements, which contain a wealth of H3K27me3 and H3K4me3 containing bivalent domains (Bernstein et al., 2006). These regions house genes responsible for encoding several transcription factors which function to maintain and regulate pluripotency and self-renewal in both early embryos and embryonic stem cells (Bernstein et al., 2006). These factors include the POU (Pit/Oct/Unc) domain-containing protein Oct-4 (also known as Pou5f1) (Nichols et al., 1998; Niwa et al., 2000; Rosner et al., 1990; Scholer et al., 1990a; Scholer et al., 1990b), the high

mobility group (HMG)-box protein Sox-2 (Avilion et al., 2003), and the homeoprotein Nanog (Chambers et al., 2003; Mitsui et al., 2003). These three transcription factors function through very distinct pathways that are thought to converge to dictate early cell fate decisions (Boyer et al., 2006). Recently, the conservation of these proteins among mammalian species has become apparent and Oct-4, Sox-2 and Nanog are considered markers of cells that have pluripotent capabilities. Also, their association with highly conserved non-coding elements suggests a conserved role for the bivalent chromatin domains mentioned above (Bernstein et al., 2006).

At this time, research has not identified bivalent domains in adult somatic MSCs from porcine adipose tissue. Analysis of these cells may reveal global methylation and/or acetylation patterns similar to their embryonic stem cell counterparts. Further characterization of porcine ASCs in this manner could assist the isolation and purification of a population of MSCs from the easily obtainable tissue of adipose that can later be tested as donor cells in nuclear transfer. Somatic cells used as donor cells in nuclear transfer typically produce more blastocyst stage embryos compared to embryos derived from embryonic stem cells (Rideout et al., 2001). About 60% of embryonic stem cells are in S-phase when used as donor cells (Rideout et al., 2001). S-phase embryonic stem cells are exposed to high levels of maturation promoting factor, which induces premature nuclear envelope breakdown and chromosome condensation. Cells likely undergo a second DNA replication cycle activating cell checkpoint mechanisms ultimately leading to inappropriate ploidy and cell cycle arrest. However, more embryonic stem cell nuclear transfer embryos develop to term than somatic cell nuclear transfer embryos (Rideout et al., 2001). Although nuclear transfer clones produced from either embryonic stem cells or somatic cells demonstrate similar phenotypic abnormalities, term embryonic stem cell nuclear transfer clones indicate that utilization of a less differentiated donor cell may be more acquiescent to appropriate reprogramming (Rideout et al., 2001).

Our laboratory has contributed to the characterization of porcine ASCs by: (1) the development of an isolation and culture procedure for both undifferentiated and differentiated (adipocytes, osteocytes, and chondrocytes) ASCs, (2) the definition of ASC growth characteristics such as cell cycle length, population doublings, (3) the confirmation of cell surface gene expression in both differentiated and undifferentiated ASCs, (4) the analysis of clonal capacity followed by differentiation potential in ASCs, (5) the determination of chromosomal stability through *in vitro* culture, (6) the analysis of stem cell surface markers through *in vitro* culture, (7) the detection of methylated DNA and acetylated histone H3 through *in vitro* culture, (8) the determination of expression patterns of chromatin remodeling proteins, and (9) the presence/absence of bivalent chromatin structures. Our overarching hypothesis is that low development rates observed following porcine nuclear transfer are a result of incomplete reprogramming of the donor cell nucleus and that somatic stem cells or those cells which demonstrate stem-like characteristics (i.e. ASCs) will be reprogrammed more completely. The new information will aid the efficiency of isolation and purification of a population of MSCs from the easily obtainable tissue of porcine adipose that can later be tested as donor cells in nuclear transfer.

Conclusions

Cutting edge methods for producing transgenic animals still need much improvement considering the inefficiency and expenses involved. By employing cells such as somatic stem cells or cells demonstrating stem-like characteristics as donor cells for nuclear transfer, resultant anomalies such as those that classify Large Offspring Syndrome may be averted and increase the success rates of nuclear transfer. Controversy associated with the use of human tissues necessitates the development of an animal model to continue research in this important area. Many treatments have already resulted from the use of tissues generated through advancements made in the porcine animal model. Therefore, the definition of stem cellular characteristics will aid in the efficient identification and isolation of a population of MSCs from

porcine adipose tissue to be later used as donor cells in nuclear transfer creating an important animal model for additional future biomedical applications.

Currently, nuclear transfer utilizing a true MSC as the donor cell is unconvincing most likely due to inefficient isolation/purification protocols and characterization. Based on the success of our preliminary experiments, we further defined cellular characteristics of MSCs derived from adipose tissue of the porcine animal model. Knowledge of the true nature and potential of MSCs will aid their contributions to medical treatments. The use of a less differentiated donor cell may be more conducive to appropriate reprogramming (Rideout et al., 2001) leading to an increased success rate in nuclear transfer which will ultimately lend to the enhancement of the number of live transgenic offspring produced. An increased efficiency will also shorten the time required to produce an adequate number of live transgenic offspring for pharmaceutical and other biomedical purposes and therefore decrease the ultimate cost of production. In turn, this will eliminate economic barriers currently blocking the application of this technology from commercial large-scale production of transgenic animals and therefore make transgenic animals a more appealing method of producing recombinant proteins.

Researchers in industry are engineering bacteria or mammalian cells into bioreactors for producing recombinant proteins for therapeutic treatments. However, these tiny bodies are incapable of producing the necessary mass quantity to adequately service the growing demand. The current shortfall hinders such therapeutic products from entering the marketplace thereby preventing patient access to new therapies, which in turn denies revenue to pharmaceutical companies who have invested large sums of money in product development. An increase in the efficiency of transgenic animal production will provide a substitute factory for the manufacture of these recombinant proteins and offer a more cost effective mechanism for the production of large quantities (i.e., kilograms).

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CHAPTER III

ISOLATION AND CHARACTERIZATION OF PORCINE ADIPOSE TISSUE-DERIVED ADULT STEM CELLS

Introduction

Stem cell applications for biomedical treatments continue to face many challenges concerning the originating source, expansion and culture, and differentiation. The controversy surrounding the use of stem cells from embryos has encouraged research of alternative stem cell sources. Numerous efforts have focused on the isolation and characterization of adult stem cells. By definition, stem cells must be able to self-renew as well as differentiate into multiple tissue phenotypes. Adult stem cells can be found among differentiated tissues. Adult stem cells can renew themselves and when removed from their niche they are able to differentiate into the multiple cell types surrounding them in order to maintain and repair that tissue or organ. The development of an animal stem cell model will enhance important biomedical treatments, such as tissue engineering and cord blood stem cell transplants utilized in human patients.

Mesenchymal stem cells (MSCs) are adult stem cells and have been isolated from many different mesodermal tissues including bone marrow (Friedenstein et al., 1976), fat (Rodbell, 1966; Rodbell and Jones, 1966) and cord blood (Broxmeyer et al., 1992). MSCs are pluripotent precursors that support hematopoiesis and formation of bone tissue, cartilage and adipose tissue (Friedenstein et al., 1976; Halvorsen et al., 2000; Gimble and Guilak, 2003). The inherent ability of MSCs to proliferate in vitro and maintain their pluripotency makes them attractive candidates for the development of an animal stem cell model.

MSCs have been isolated from various tissues based on their ability to adhere to plastic and their morphology similar to that of fibroblasts (Luria et al., 1971; Kassem et al., 1993; Rickard et al., 1996). However, hematopoietic cells contaminate MSCs creating a heterogenous population of cells in culture. Cell surface markers, such as CD9, CD10, CD13, CD29, CD34, CD44, CD49 d, CD49 e, CD54, CD55, CD59, CD105, CD106, CD146, CD166

(Gronthos et al., 2001), SSEA-4 (Gang et al., 2007) and a combination of others, have been utilized to identify and isolate MSCs from these contaminating hematopoietic cells as well as to further characterize MSCs. Gene and protein expression profiles have been defined in some human bone marrow and adipose tissue-derived MSC populations showing the presence of these cell surface markers (Simmons and Torok-Storb 1991; Barry et al., 1999; Gronthos et al., 2001). In addition to cell surface markers, another important characteristic used to distinguish MSCs from hematopoietic cells is their ability to support differentiation into several different cell types depending on culture conditions.

Human adipose tissue-derived adult stem (ASCs) cells are self-renewing, can be differentiated into multiple tissue lineages and exhibit a high expression level of CD34, CD44 and other stem cell markers (Gronthos et al., 2001; Guilak et al., 2006). At this time, these stem cell characteristics have not been identified in porcine ASCs. In the present study, a protocol has been developed for the isolation and culture of porcine ASCs. Growth characteristics including population doublings, cell cycle length and proliferative capacities have been determined. We also examined these stem cell-like cells for the capacity to self-renew and differentiate into cell lineages.

Materials and Methods

All reagents were purchased from Sigma (St. Louis, Mo., USA) unless otherwise stated.

Establishment of Primary Cultures

Subcutaneous fat (10 g per pig) was harvested from the dorsal abdominal area in mature female crossbred domestic pigs (n = 5) between the age of 6 months and 1 year of age at a local abattoir. Samples were transported on ice in Dulbecco's phosphate-buffered saline (dPBS; Gibco, Grand Island, N.Y., USA) containing 200 U/ml of penicillin and 200 µg/ml of streptomycin (Gibco) and 2.5 µg/ml of Fungizone (Gibco). The tissue was finely minced and washed with dPBS containing P/S and Fungizone. Each sample was digested with an equal volume of 0.1% collagenase Type I (240 IU/ml; Gibco) in dPBS with 1% bovine serum albumin

in a shaker incubator rotating at 200 rpm at 39° C for 2 hours. The cell suspension was filtered through a dual filter system comprised of two nylon net filters (20 and 180 µm; Millipore, Bedford, MA, USA). Adipocytes were separated from the stromal-vascular fraction by centrifugation at 1,200 rpm for 5 minutes. Cells were then stained with Hoechst 33342 (2 µg/ml) to determine the number of nucleated cells in the suspension. Cells were plated at 6,700 nucleated cells/cm² in 10 ml of expansion medium containing Dulbecco's modified Eagle medium (DMEM; Hyclone, Logan, UT, USA) with high glucose supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 U/ml of penicillin and 100 µg/ml of streptomycin and 2.5 µg/ml of Fungizone in a 75-cm² culture flask and incubated at 39°C in 5% CO₂.

Cell Culture Maintenance

Fresh medium was supplied every 3 to 4 days. At 70 to 90% confluence, adipose cells were passaged by trypsinization, counted using a hemacytometer and re-seeded at an initial concentration of 6,700 cells/cm². Population doublings were calculated at each passage using the following equation: $\log (\text{final concentration}/\text{initial concentration}) \times 3.33$ (Arat et al., 2006). Also, cell cycle length was calculated based on the following equation: number of days in culture/population doublings. Cells were passaged until replicative senescence was observed. Replicative senescence was observed when the cells ceased to divide and was defined by a change in morphology from an elongated, spindle like to flattened, irregular shape with dispersed cytoplasm and the appearance of cytoplasmic vacuoles.

Cloning Capacity Procedures

Cells were isolated according to the above procedure and plated from the initial culture (passage 0) at approximately 5 cells/cm² in 10 ml of expansion medium in a 100-mm culture dish and incubated at 39°C in 5% CO₂. Adherent cells formed colonies after 10 days in culture and were selected for ring cloning (passage 0) based on the following criteria: colonies contained at least 100 cells and colonies were located a minimum of one microscopic field from all other colonies. Cells were passaged by trypsinization and re-seeded in a 1.9-cm² culture

dish (passage 1) and at 70 to 90% confluence passaged into a 9.62-cm² culture dish (passage 2), a 25-cm² culture flask (passage 3) and a 75-cm² culture flask (passage 4).

Differentiation of Primary Cultures and Clones

At 70 to 90% confluence (following passage 4 for clones), cells were passaged by trypsinization and cultured for 21 days in culture medium previously demonstrated to induce differentiation (Zuk et al., 2002; Gimble and Guilak, 2003). Culture media were changed every 3 to 4 days throughout the study. For adipogenesis, cells were cultured on days 1 to 4 in differentiation medium containing DMEM supplemented with 3% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin, 2.5 µg/ml of Fungizone, 10 ng/ml of insulin, 10⁻⁹ M, dexamethasone, 250 µM isobutyl methylxanthine and 0.2 mM indomethacin. Culture medium beyond day 4 contained DMEM with 3% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin, 2.5 µg/ml of Fungizone, 10 ng/ml of insulin and 10⁻⁹ M dexamethasone.

For chondrogenesis, cells were cultured for the duration of differentiation in medium containing DMEM with 10% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin, 2.5 µg/ml of Fungizone, 6.25 µg/ml of insulin, 10 ng/ml of transforming growth factor-β and 10⁻⁹ M dexamethasone.

For osteogenesis, cells were cultured in differentiation medium containing DMEM with 10% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin, 2.5 µg/ml of Fungizone, 10 mM β-glycerophosphate, 50 ng/ml of ascorbic acid and 10⁻⁹ M dexamethasone.

Histological staining techniques were used to determine differentiation. Nile Red, a stain, which detects intracellular lipid accumulation, was used to determine adipocyte differentiation. Safranin O was utilized to identify glycosaminoglycans in the extracellular matrix of differentiated chondrocytes. After osteogenic induction, Alizarin Red S was employed to detect mineralized calcium deposition.

Experimental Design

Cell lines were defined as cells isolated from a single porcine donor. Five cell lines were cultured and population doublings and cell cycle length were calculated at each passage (except for clones). Cells were trypsinized and re-seeded at 30 to 40% confluence to avoid contact inhibition and possible differentiation. Passaged lines were cultured until 70 to 90% confluence was reached. Two replicates per cell line were performed for establishing growth characteristics and one replicate per cell line was performed for cloning procedures.

Results

Isolation of Cell Lines

Porcine adipose tissue-derived adult MSC lines were successfully established from 5 female pigs. The average number of mononuclear cells was $2.7 \times 10^6/10$ g for each fat tissue sample. Of those mononuclear cells, an estimated 26% adhered to the plastic surface of the culture vesicle, while the rest were removed during the first medium change after the first 24 hours of culture. Adherent cells began forming colonies beginning on day 1 after the primary culture was established. Most cells in culture demonstrated an elongated, spindle-like fibroblastic morphology (Figure 3.1, black arrow), while others displayed a more rounded appearance (Figure 3.1, white arrow). Cell cultures expanded rapidly to 70 to 90% confluence by days 8 to 10 after initial seeding.

Growth Characteristics

Population doublings were calculated following the first passage according to the calculation described above. From two cell lines, two replicates of each line were analyzed in parallel. Cumulative population doublings were measured for each replicate with a mean of 29 population doublings without reaching replicative senescence (Figure 3.2 A). Average cell cycle lengths for adherent, undifferentiated cells were recorded throughout the duration of the culture interval. Cells completed one cell cycle approximately every 3.3 days (Figure 3.2 B). Cells did not reach replicative senescence by the end of this study (100 days).

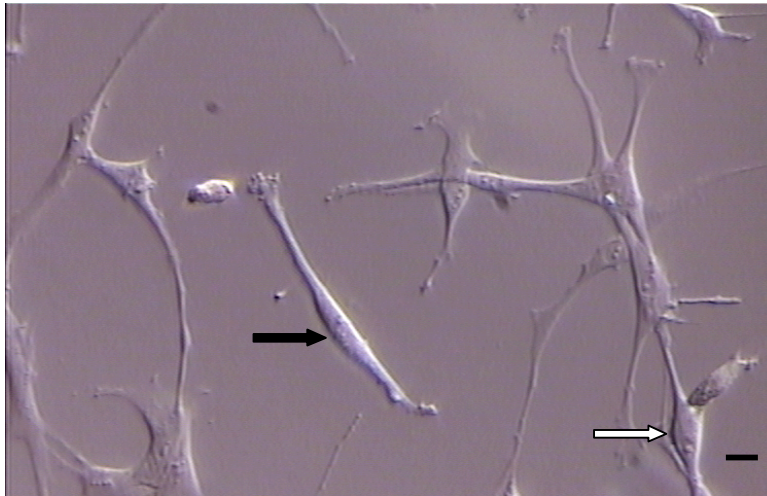


Figure 3.1. Undifferentiated stromal cell morphology. Discrete colonies demonstrating spindle-like fibroblastic morphology adhered to plastic within 24 hours of initial seeding. Most cells displayed a characteristic elongated shape (black arrow), while others had a more rounded appearance (white arrow). Magnified using a 20X objective.

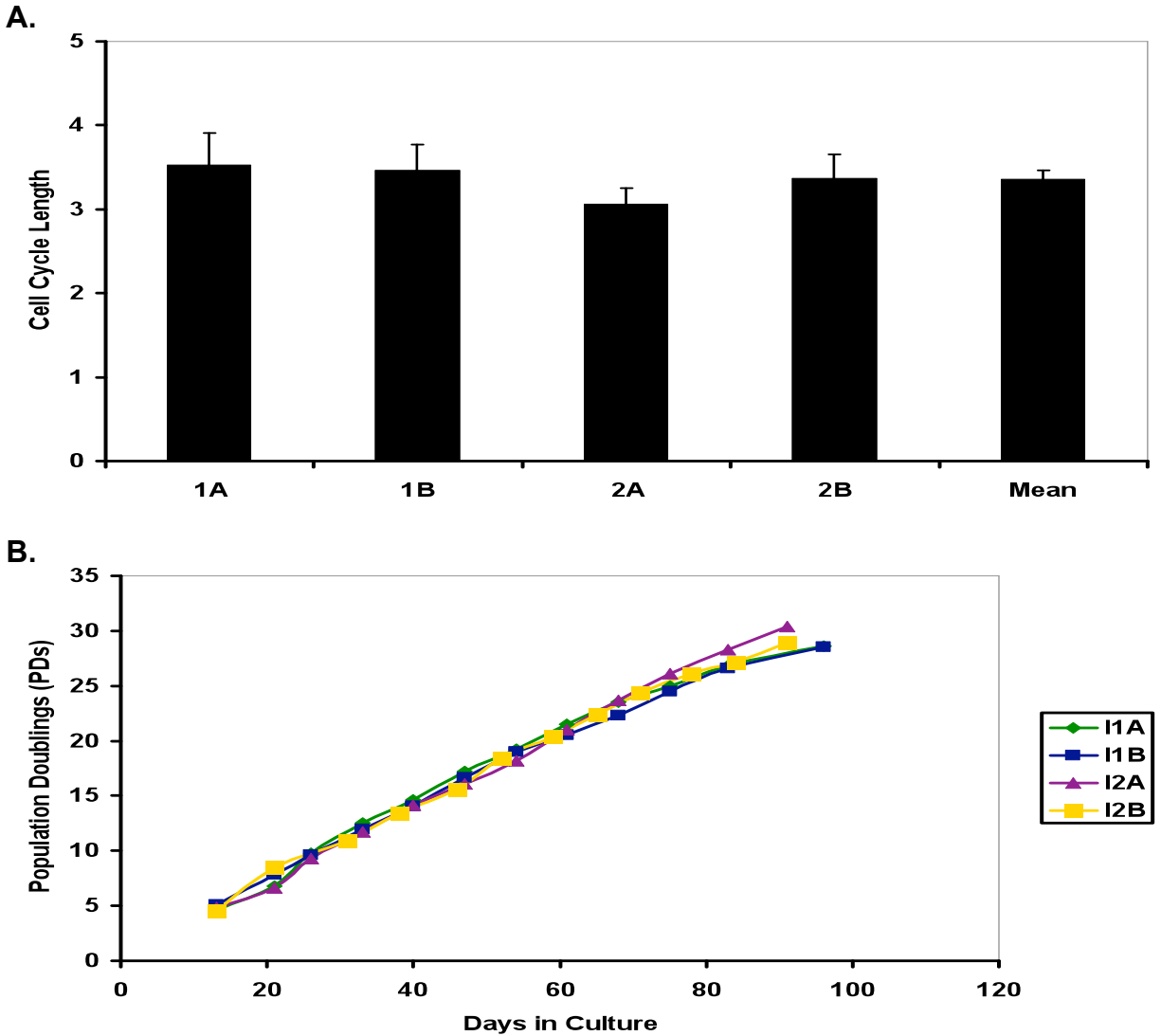


Figure 3.2. Proliferative characteristics of undifferentiated stromal cells: 1 and 2 represent each cell line while A and B are replicates. (A) Average cell cycle lengths were recorded throughout the duration of culture. (B) Proliferative capacity indicates cells have not reached observed, replicative senescence.

Clonal Analysis of Porcine ASCs

A total of 16 clones were selected for ring cloning from each of 2 donor pigs and 15 from the 3rd donor resulting in 47 attempted clones selected in all. Of the 47 clone attempts, 8 did not produce a clone for passage 1 (17.0%) and replicative senescence was observed in 12 of 47 clones (25.5%). There were 7 (43.7%) surviving clones produced from the first donor that were successfully cultured to passage 4, the second donor produced 10 (62.5%) and there were 14 (87.5%) from the third donor. Cloned colonies expanded in a monolayer maintaining a fibroblastic morphology throughout the culture interval. Of the 31 cloned colonies that survived to passage 4 without reaching replicative senescence or acquiring contamination, 19 colonies (62.0% of the total cloned population) were subjected to multiple cell lineage differentiation analysis (Table 3.1).

Cell Lineage Differentiation of Cloned Porcine ASCs

Clonal cell cultures were exposed to differentiation conditions. On day 21 of differentiation, stains specific for characteristics of differentiated adipocytes, osteocytes and chondrocytes were utilized. Nile red, a stain which detects intracellular lipid accumulation, was used to determine adipocyte differentiation. After chondrogenic induction, safranin O was used to detect glycosaminoglycans in the extracellular matrix. Alizarin red S identified mineralized calcium deposition in differentiated osteocytes. After 21 days of culture in differentiation medium, a qualitative estimate was assigned to each differentiation condition based on multiple fields of view.

Of the 19 cloned colonies subjected to differentiation conditions, 92% of colonies differentiated under adipogenic conditions stained positive for intracellular lipids (Figure 3.3 A) and the undifferentiated control cells displayed expression of these intracellular lipids, only less intensely (Figure 3.3 C). Chondrogenic induction was detected in 97% of cloned colonies by the presence of glycosaminoglycans in differentiated clones (Figure 3.3 E). This was also noted in undifferentiated cell populations, but again less intensely (Figure 3.3 F). Finally, osteocyte

Table 3.1. Number of surviving porcine clones to passage 4 and differentiation

Cell line	Total # clones	P1	P2	P3	P4*	Diff culture	[†] Adipogenesis	[†] Chondrogenesis	[†] Osteogenesis
I	16	16	12	12	7	0	0 (0%)	0 (0%)	0 (0%)
II	16	16	13	13	10	5	10/10 (100%)	10/10 (100%)	10/10 (100%)
III	15	15	14	14	14	14	25/28 (89%)	27/28 (96%)	28/28 (100%)

P Passage; Diff = Differentiated.

*Approximately 14 population doublings or 4-8 weeks in culture.

[†]Two replicates.

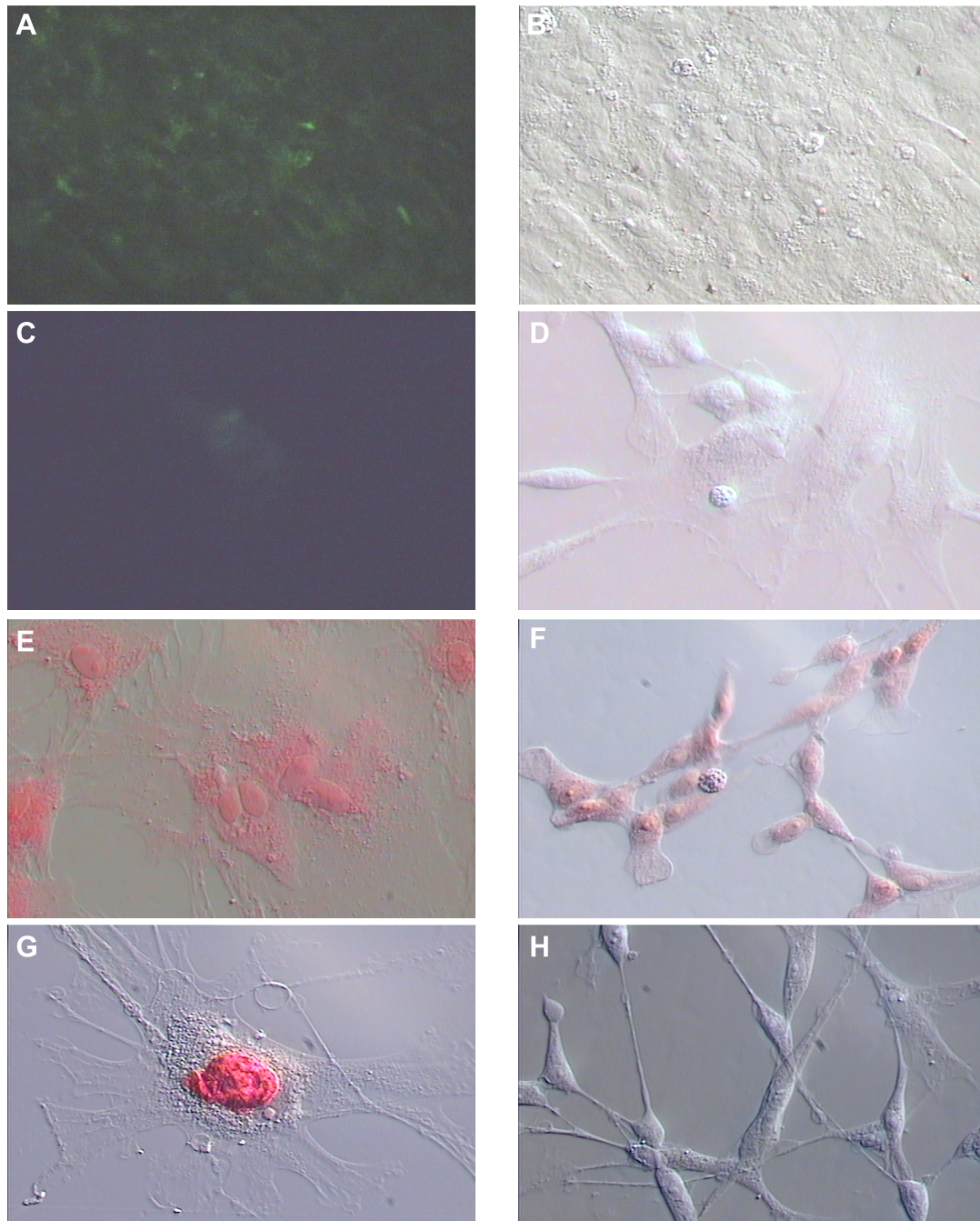


Figure 3.3. Differentiation of P4 clones. Adipogenic differentiation stained positive with Nile Red staining; viewed with a FITC filter (A) and phase contrast (B). Undifferentiated stromal cells (control) demonstrated only slight Nile Red staining (Panel C-FITC filter and Panel D-phase contrast). Chondrogenic induction was detected by Safranin O in differentiated clones (E) and less intensely in undifferentiated cell populations (F). Osteocyte differentiation was observed only in induced cells (G) and not in undifferentiated stromal cells (H). Images A-F and H were magnified using a 20X objective while image G was magnified using a 40 X objective.

differentiation was observed in 100% of cloned colonies. Positive staining for calcium deposition was observed only in the induced cells (Figure 3.3 G) and not in the undifferentiated stromal cells (Figure 3.3 H).

Discussion

Stem cell biology has applications in regenerative medicine for repairing, replacing or regenerating diseased tissues and organs. Stem cells must have the capacity for self-renewal and must have the ability to give rise to differentiated cell types to be considered 'stem cells'. The use of somatic or adult stem cells, found in adult tissues, escapes the controversy associated with the use of stem cells derived from embryos and fetal tissues. Also, adult stem cells have the potential to elude immunological rejection and disease contraction when transplanted back into the donor (Merok et al, 2001). The advancement of stem cell technology is already contributing to applications, such as gene therapies for disease and the development of research techniques, such as bone marrow transplants and transgenic mice (Merok et al., 2001).

The emerging concept of plasticity (also known as transdifferentiation) in stem cells suggests that somatic stem cells have the ability to dedifferentiate from their source cell type and then differentiate into new cell types (Guilak et al., 2004). The improvement of culture conditions and the definition of cellular characteristics for the efficient isolation and purification of a population of MSCs from multiple species-specific tissues will likely create important animal models for additional biomedical applications.

At this time, knowledge of the optimal culture conditions for pluripotent and differentiated MSCs from adipose, bone marrow and cord blood is limited. Also, culture characteristics of these cells have not been clearly defined and studies generating gene and protein expression profiles are underway, but remain inconclusive. This study has developed a successful protocol for the isolation and culture of porcine ASCs. We were able to establish primary MSC lines from 5 female pigs isolating an average of 2.7×10^6 MSCs/10 g from each fat tissue sample. Similar

quantities of cells have been isolated from adipose tissue of rhesus monkeys and humans (Izadpanah et al., 2006; Oedayrajsingh-Varma et al., 2006). Of the cells isolated, an estimated 26% adhered to the plastic surface of the culture vesicle. Most cells in culture displayed an elongated, spindle-like fibroblastic morphology. Cells were plated at a density of 6,700 cells/cm², while lower concentrations did not render rapid colony cultivation (data not shown), suggesting that the cells prefer to have neighboring cells within a closer range. Growth characteristics of these MSCs indicate that a mean of 29 population doublings could be reached without replicative senescence. These cells completed one cell cycle approximately every 3.3 days and had not yet reached replicative senescence by the end of this study (100 days).

In comparison, Zuk et al. (2001) described human adipose tissue to harbor approximately 2–6 x 10⁶ cells per 300 ml of processed lipoaspirate which can be maintained in culture through passage 13 (approximately population doubling 22) or 165 days. Human ASCs isolated by resection or tumescent liposuction demonstrate a cell cycle length of approximately 1.5–2.8 days (Zuk et al., 2001; Oedayrajsingh-Varma et al., 2006). Also, human adipose tissue isolated using these techniques contained adherent cells representing 1.9 to 6.3% of the total cellular population (Oeday rajsingh-Varma et al., 2006). Porcine bone marrow derived MSCs can be isolated with a yield of approximately 2.3 x 10⁸ cells per 20 ml bone marrow aspirate (Bosch et al., 2006). Plating 500 bone marrow-derived cells per cm² renders approximately 4,000 cells per cm² after 3 to 4 days in culture (estimating a cell cycle length of 0.75 to 1 day) (Zeng et al., 2006). Also, these cells can be cultured for more than 100 population doublings (Zeng et al., 2006). Based on these findings, growth characteristics of the porcine ASCs used in this study demonstrate similar results.

Clonal analysis indicates that these stem cell-like cells have the capacity to self-renew and differentiate into cell lineages much like human ASCs (Guilak et al., 2006). We hypothesize that the observed replicative senescence in these cloned colonies is a result of the cellular cloning processes because primary cultures did not exhibit this phenomenon. Colonies

subjected to adipogenic, chondrogenic and osteogenic differentiation conditions all stained positive. While undifferentiated controls (approximately population doubling 30) stained positive for adipogenesis and chondrogenesis, it is evident that the colonies subjected to differentiation conditions stained much more intensely than colonies that were not subjected to differentiation conditions.

Improved culture conditions and defined cellular characteristics of these porcine ASCs have been identified. The techniques used in this study will increase the efficiency of isolation and purification of a population, of stem cell like cells from easily obtainable tissues. The isolation and characterization of a somatic stem cell population from a model animal, such as the pig, could prove valuable in tissue engineering research. The availability of such an animal model will be very beneficial in determining the true efficacy of somatic stem cell tissue, regeneration versus the use of embryonic stem cells.

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CHAPTER IV

CELL SURFACE CHARACTERIZATION AND KARYOTYPIC ANALYSIS OF PORCINE ADIPOSE-DERIVED STEM CELLS

Introduction

Over the last twenty years, human bone marrow stromal cells have been shown to differentiate along adipocyte, osteocyte and other mesodermal pathways as well as support the proliferation of hematopoietic stem cells. It has been shown that specific adhesion molecules expressed on the surface of bone marrow stromal cells mediate the cell-cell interactions supporting differentiation and proliferation (Kincade et al., 1988). The presence of these molecules may be utilized in isolation protocols to identify and enhance the purification of a population of cells within a heterogeneous population.

Human adipose tissue is an alternative source of multipotent stromal cells, which is largely derived from processed liposuction aspirates. Human and porcine adipose tissue–derived adult stem cells (ASCs) are a self-renewing population of cells with a multilineage plasticity similar to bone marrow–derived mesenchymal stem cells (MSCs) and show promise for use in combination with various biomaterials for reconstructive tissue engineering (Halvorsen et al., 2001a; Hauner et al., 1989; Williams et al., 2008). The phenotypic profile of human ASC surface proteins has been partially characterized for stem cell-associated cluster differentiation molecules including CD9, CD10, CD13, CD29, CD31, CD34, CD44, CD49_a, CD49_d, CD49_e, CD54, CD55, CD59, CD63, CD73, CD90, CD105, CD106, CD144, CD146, CD166 (Gronthos et al., 2001; Mitchell et al., 2006), and for SSEA-4 (Gang et al., 2007), ABCG2, ALDH, VEGFr-2 (Mitchell et al., 2006) and a combination of other molecules. However, the surface protein phenotype has not been described for porcine ASCs at this time.

Multiple research groups have characterized human ASCs by describing the surface phenotype, however, there are many subtle discrepancies throughout the literature. The effects of these differences on the identity of these cells remain unknown. Various factors may play a

role in producing these inconsistencies. For example, porcine ASCs are isolated from fat depots likely different from human ASC liposuction aspirates. Also, the age of the donor may be different and cause the results to disagree. In addition, different isolation procedures, the specific cell seeding density, differences in culture media, culture duration, the cell cycle, the passage numbers analyzed, whether the cells were cryopreserved prior to analysis and even the exposure to plastic (Planat-Benard et al., 2004; Rehman et al., 2004) all may cause incongruent results. Therefore, it is important to characterize porcine ASCs on the cell surface level.

The inherent ability of mesenchymal stem cells such as porcine ASCs to proliferate *in vitro* and maintain their aptitude for pluripotency (Williams et al., 2008) makes them attractive candidates as donor cells for more efficient reprogramming during nuclear transfer (NT) compared to somatic cells currently in use. Studies have however generated contradicting results for the success of stem cells in NT; for example, skin-derived stem cells increased NT efficiency (Hao et al., 2009; Zhu et al., 2004) while hematopoietic stem cells did not (Inoue et al., 2006; Sung et al., 2006). Porcine ASCs have not been tested as donor cells for NT at this time.

Successful NT in combination with applicable genetic modifications must be conducted using normal diploid cells before they reach senescence (Denning et al., 2001). Primary cells utilized to establish culture possess the capacity to survive only a finite number of population doublings (PDs) before cellular senescence is reached (Hayflick and Moorhead, 1961). As cells approach this limit, the rate of proliferation decreases (Cameron, 1972) and the probability of cells to undergo cytokinetic abnormalities and chromosomal alterations increases substantially (Giraldo et al., 2006; Oback and Wells, 2002). The current techniques employed today to establish and maintain primary *in vitro* cell cultures may also be responsible for the induction of these anomalies (Chu, 1962; Denning et al., 2001; Kasinathan et al., 2001; Oback and Wells, 2002; Rubin, 1997). Spontaneous transformations, which will also influence the success of NT,

have been described in human and murine adipose tissue-derived MSCs (Rubio et al., 2005) and have been reported to accumulate proportionally to the time in culture (Oback and Wells, 2002). However, other studies indicate that human bone marrow and adipose tissue-derived MSCs do not mutate and maintain chromosomal configuration through culture (Bernardo et al., 2007; Meza-Zepeda et al., 2008).

Porcine ASCs have not been characterized in terms of chromosomal stability through *in vitro* culture. Chromosomal aberrations and a limited proliferative capacity of somatic donor cells cultured *in vitro* dramatically influence the success of NT (Kasinathan et al., 2001). The manifestation of genetic modifications in donor cells for NT requires a significant number of PDs and, unfortunately, these deleterious effects are impossible at this time to circumvent. But the identification of a donor cell that is genetically stable for a long period of time *in vitro* may be the key to increasing the efficiency of NT.

The current study partially characterized the surface protein phenotype of undifferentiated porcine ASCs in comparison with the immunophenotype of undifferentiated human ASCs as reported in the literature using flow cytometry and enhanced chemiluminescence Western blot analysis. Also, the chromosomal stability of porcine ASCs was determined through *in vitro* culture in order to further characterize them as potential donor cells for NT. With a more complete characterization of porcine ASC (i.e. stem cell surface markers, chromosomal stability) a donor cell population, which can be more efficiently reprogrammed following fusion with the oocyte, may be identified.

Materials and Methods

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Establishment of Primary Cultures and Cell Culture Maintenance

Primary cultures were established following procedures previously documented (Williams et al., 2008). Briefly, 10 g subcutaneous fat was harvested from the dorsal abdominal

area in mature female crossbred domestic pigs (n = 20) between the ages of 6 months and 1 year of age at a local abattoir. The tissue was finely minced and washed with Dulbecco's phosphate-buffered saline (dPBS) containing 200 U/ml of penicillin and 200 µg/ml of streptomycin (P/S) and 2.5 µg/ml of Fungizone (Gibco). Each sample was homogenized with an equal volume of 0.1% collagenase Type I (240 IU/ml; Gibco) in dPBS with 1% bovine serum albumin (BSA) in a shaker incubator rotating at 200 rpm at 39°C for 2 hours.

The cell suspension was filtered through a dual filter system comprised of two nylon net filters (20 µm and 180 µm; Millipore, Bedford, MA, USA). Cells were then stained with Hoechst 33342 (2 µg/ml) to determine the number of nucleated cells in the suspension. Cells were plated at 6,700 cells/cm² in 10 ml of expansion medium containing Dulbecco's Modified Eagle Medium (DMEM; Hyclone, Logan, UT) with high glucose supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 U/ml of penicillin and 100 µg/ml of streptomycin and 2.5 µg/ml of Fungizone in a 150-cm² culture flask and incubated at 39°C in 5% CO₂.

Fresh medium was supplied every 3 to 4 days. When 70 to 90% confluence was achieved, the adipose cells were harvested by trypsinization, counted using a hemacytometer and re-seeded at an initial concentration of 6,700 cells/cm². Cells were passaged until replicative senescence was observed.

Stem Cell Surface Protein Immunolabeling

Porcine ASCs were harvested by trypsinization, washed and resuspended in cold PBS containing 10% FBS and 1% sodium azide. The cells were incubated for 1 hour at room temperature in primary antibodies (10 µg/ml) to hβ-Microglobulin (human, BD Pharmingen, San Jose, CA, USA, Catalog # 555550), pCD3 (porcine, direct label, BD Pharmingen, San Jose, CA, USA, Catalog # 559582), pCD8 (direct label, BD Pharmingen, San Jose, CA, USA, Catalog # 551303), pCD29 (BD Pharmingen, San Jose, CA, USA, Catalog # 552369), pCD44 (Abcam, Cambridge, MA, USA, Catalog # ab19622), hCD90 (BD Pharmingen, San Jose, CA, USA, Catalog # 555593) pCD117 (AbD Serotec, Catalog # MCA2598), SSEA-4 (Developmental

Studies Hybridoma Bank, Iowa City, IA, USA, Raleigh, NC, USA, Catalog # MC-813-70) and mouse Sca-1 (Ly-6A/E, BD Pharmingen, San Jose, CA, USA, Catalog #557403).

For indirect labeling, cells were then incubated at room temperature for 30 minutes in 10 µg/ml of the Alexa-Fluor® 488 (Invitrogen, Eugene, OR, USA) conjugated secondary antibody. Cells were counterstained with propidium iodide (50 µg/ml) for 15 minutes. The cells were then washed and resuspended for flow cytometric analysis at a concentration of 1,000,000 cells/ml in cold PBS containing 10% FBS and 1% sodium azide.

Flow Cytometric Analysis of Stem Cell Surface Proteins

Samples were analyzed for relative percent positive events for each stem cell surface proteins using the Accuri® C6 Flow Cytometer® (Accuri Cytometers, Ann Arbor, MI, USA). Labeled cells were excited with a 488 nm diode laser and a 488 nm diode laser was used to detect PI levels (FL3-channel). The DNA content derived from the PI levels was used to distinguish live cells from dead cells in the FL3-channel. Percent positive events for stem cell surface proteins were detected at >670 nm in the FL1-channel. A gate was established to exclude non-specific binding as a whole based on secondary antibody only staining. FL3 and FL1 dot plots were generated using the Accuri CFlow Plus® (Accuri Cytometers) software and quadrants were instated to distinguish dead from live cells and immunolabeled cells from unstained cells. Approximately 30,000 to 100,000 events were recorded per sample at early and late population doublings.

Western Blot Analysis of Stem Cell Surface Proteins

Early PD human (positive control) and porcine ASCs were lysed and proteins extracted using a solution containing the M-PER® Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA), Halt™ Protease Inhibitor Single-use Cocktail (Thermo Scientific) and 10 µl/ml EDTA solution on a rotator for 10 minutes. Cellular debris was removed by centrifugation at 14,000 x g for 15 minutes. The protein extractions were analyzed by a typical western blot procedure. Briefly, protein extractions were separated by SDS-polyacrylamide gel

electrophoresis on 10% Tris-HCl Ready Gels (Bio-Rad, Hercules, CA, USA). Extractions were then transferred to an Immun-Blot™ polyvinylidene fluoride membrane (Bio-Rad) and hybridized using the following primary antibodies: hCD44 (BD Pharmingen, San Jose, CA, USA, Catalog # 550988), hCD90 (BD Pharmingen, San Jose, CA, USA, Catalog # 555593), pCD44 (Abcam, Cambridge, MA, USA, Catalog # ab19622), pCD117 (c-Kit; AbD Serotec, Raleigh, NC, USA, Catalog # MCA2598), and β -actin (Abcam, Cambridge, MA, USA, Catalog # ab8226) for a loading control. The secondary antibody to each primary was conjugated with horse radish peroxidase (Bio-Rad) and an enhanced chemiluminescence (ECL) kit (Amersham, Little Chalfont, Buckinghamshire, UK) was used for detection via exposure to x-ray film.

Karyotypic Analysis

At each passage analyzed (starting at passage 2), an additional 75 cm² culture flask was seeded with approximately 6,700 ASCs/cm². Then 24 hours after plating, adherent cells were incubated in a 0.05 μ g/ml KaryoMAX® Colcemid® solution (Gibco) at 37°C for approximately 30 minutes. Cells were exposed to this low dosage of Colcemid® for only 30 minutes to reduce the risk of the induction of aneuploidies. The synchronized cells were harvested by enzymatic dissociation using trypsin and then resuspended and incubated in a 0.9% sodium citrate hypotonic solution for 5 minutes in a 37°C waterbath. Swelled cells were then washed and stored in fixative containing a 1:3 ratio of acetic acid:methanol at -20°C.

For slide preparation, the cells were washed in fresh, cold fixative and several drops were placed on pre-cleaned glass slides (Gold Seal® Products, Portsmouth, NH, USA), which were allowed to dry at room temperature for 10 minutes. The slides were then flooded with a fixative solution of 1:1 acetic acid:methanol and allowed to dry again at room temperature for 10 minutes. The chromosomes were stained in 0.4% Accustain® Geimsa Stain (Sigma) for 15 minutes, rinsed with deionized water and air-dried at room temperature.

Metaphase spreads were observed using bright field microscopy under 100x oil immersion. Spreads were selected only if chromosomes could be visibly distinguished from

each other and were observably derived from a single cell. A digital image was captured for each metaphase spread using the QCapture Pro imaging software (QImaging, Surrey, BC, Canada). To avoid double counting, a digital mark was placed on each chromosome in each picture using the Rincon™ (ImagingPlanet, Goleta, CA, USA) image analysis software. For each cell line, 20 to 30 metaphase spreads were analyzed per passage. A total of 714 metaphase spreads were analyzed for this study.

Experimental Design

For flow cytometric analysis, a total of fourteen primary cell lines were established and cultured until cells reached senescence. At each passage analyzed, cells were trypsinized and re-seeded at 30 to 40% confluence to avoid possible contact inhibition and differentiation. Passaged lines were cultured until 70 to 90% confluence was reached. A portion of cell lines were analyzed for selected stem cell surface markers at early passages (PD 1-10) and late passages (PD 11-34). For western blot analysis, total protein was extracted from three early PD (<PD 3) cell lines. ECL-western blot analysis was performed on all three protein samples; PVDF membranes were cut to separate and incubated accordingly for each stem cell surface protein. The human protein extraction was derived from a PD 1 human ASC cell line and used as a positive control for the human antibodies (CD44 and CD90) and to test for antibody cross-reactivity for the porcine antibodies (CD44 and CD117). Representative exposed x-ray films are presented.

For karyotype analysis, two ASC cultures were established from each of three primary cell lines and were cultured under the exact same conditions. At each passage analyzed, one culture was used to prepare metaphase spreads and the other was used to subculture the cell line for continuous karyotype analysis at subsequent passages. Percent aneuploidies were calculated at population doublings (PD) 1-10, PD 11-20 and PD 21-30.

Statistical Analysis

In this study, Chi-square analysis was employed to karyotypic data in order to determine statistical differences between cell lines (pASC 1, pASC 2 and pASC 3) and PD (PD 1-10, PD 11-20 and PD 21-30). For this test, a $P < 0.05$ was considered significant. Data were analyzed using the SigmaStat Statistical Software Version 3.5 (Systat Software, Inc., San Jose, CA, USA).

Results

Flow Cytometric Analysis of Stem Cell Surface Proteins

Flow cytometric analysis was performed on multiple pASC cell lines at early population doublings (PD; PD 0-10) and at late PDs (PD 11-34) for the detection of the presence of various stem cell surface markers. Results demonstrate that profiles are similar for some cell surface markers and not similar for other markers on the human ASC surface as reported in the literature (Gronthos et al., 2001; Katz et al., 2005; Lo Cicero et al., 2008). Analyses of porcine ASCs at early PDs 0-10 demonstrated essentially no positive events for CD44 or the lectin embryonic stem cell surface marker SSEA-4 (Table 4.1; Figure 4.1), whereas these proteins were, in fact, found on the surface of human ASCs (Gronthos et al., 2001; Katz et al., 2005; Lo Cicero et al., 2008). In this study, we also did not detect the presence of CD3, CD8, CD117 (c-Kit) or stem cell antigen-1 (Sca-1; Table 4.1; Figure 4.1). The lack of CD8 and CD117 was consistent with the results for these proteins in human ASCs (Katz et al., 2005). At this time, there is no literature to report the presence of CD3 or Sca-1 proteins on the human ASC surface. However, the percentage of positive events for CD29 (4.74 ± 2.37 ; range 0.1 – 10.5) and CD90 (12.94 ± 7.06 ; range 0 – 38.6) expression was found to be elevated and more comparable to those values reported in the literature (Table 4.1; Figure 4.1). The mean percent positive values generated for CD29 and CD90 are, however, lower than that reported for human ASCs. It is important to note that the antibody used for CD90 was directed against a human antigen and cross reactivity between the pig and the human species may not have occurred.

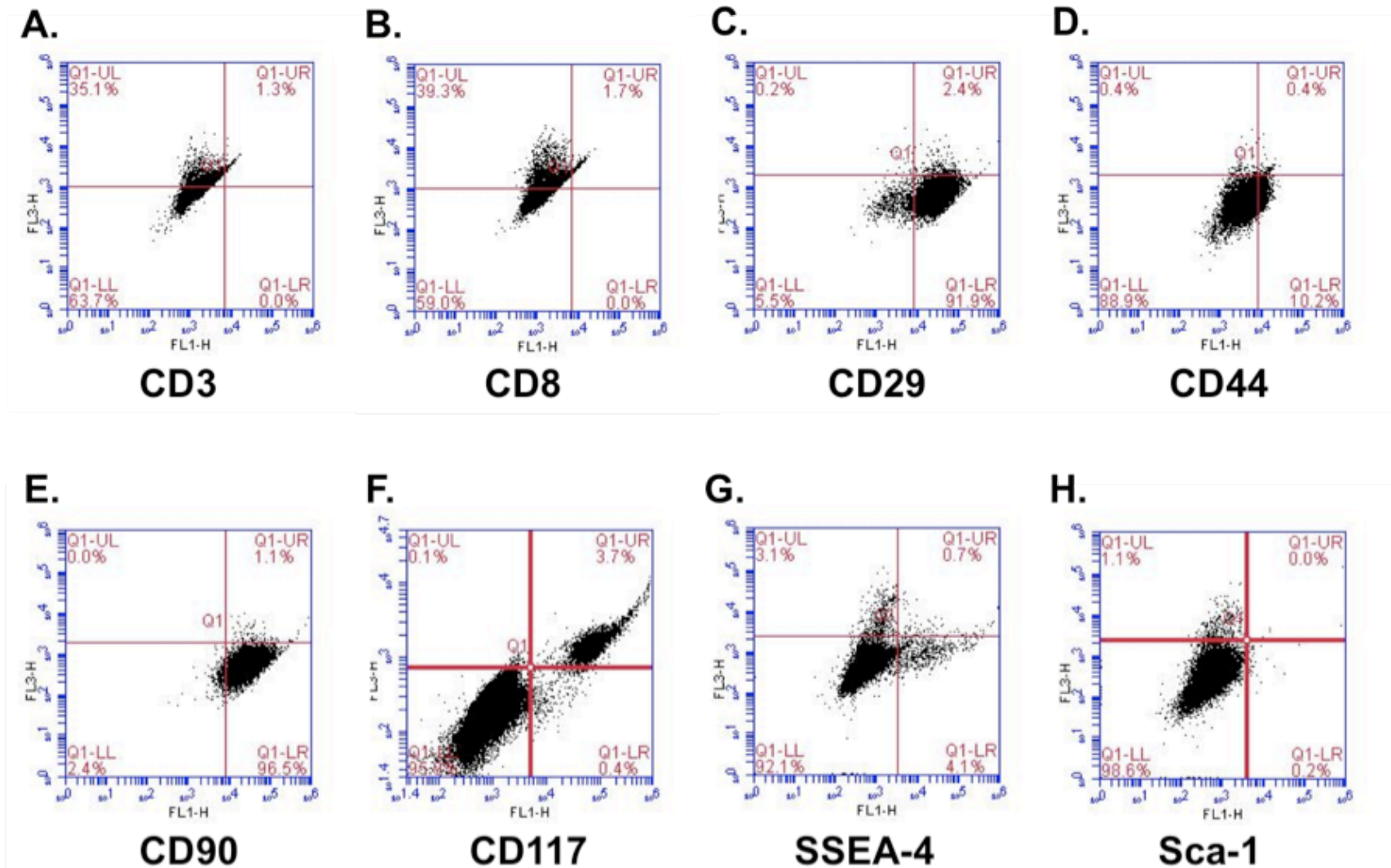


Figure 4.1. Immunophenotypic analysis of porcine ASC surface proteins. Representative dot plots for CD3 (A), CD8 (B), CD29 (C), CD44 (D), CD90 (E), CD117 (F), SSEA-4 (G) and Sca-1 (H). Dot plots represent porcine ASCs cultured at early PDs 0-10. Quadrants were set according to fluorescence signals generated from propidium iodide and Alexa-Fluor® 488 and the percentage of live cells positive for each stem cell antigen is depicted in the lower right corner.

Table 4.1. Positive Events for Early Population Doublings 0-10

Surface Marker	# Cell Lines Analyzed	Mean % Positive \pm SEM	Range (%)	[‡]Human ASC % Positive
pCD3	3	< 1	0.1 – 0.2	NP
pCD8	3	0	0	0 ^{††}
pCD29	5	70.58 \pm 13.25	33.9 – 92.8	98.1 \pm 1 [†]
pCD44	6	6.98 \pm 1.73	4.4 – 10.2	60 \pm 15 [†]
hCD90	5	86.14 \pm 5.66	69.5 – 96.5	25.96*
pCD117	5	1.00 \pm 0.66	0.1 – 3.6	0.2 \pm 0.4 ^{††}
SSEA-4	3	< 1	0.2 – 1.1	+**
mSca-1	3	< 1	0.1 – 0.2	NP

p = Porcine, h = Human, m = Mouse, NP = Not Published.

[‡]All human ASCs cells analyzed were early passages.

[†]Gronthos et al., J Cell Phys 2001; 189:54-63.

*Zuk et al., Mol Bio Cell 2002; 13:4279-4295.

^{††}Katz et al., Stem Cells 2005; 23:412-423.

**Lo Cicero et al., Cell Prolif 2008; 41:460-473.

Table 4.2. Positive Events for Late Population Doublings 11-34

Surface Marker	# Cell Lines Analyzed	Mean % Positive \pm SEM	Range (%)	[‡]Human ASC % Positive
pCD3	3	< 1	0 – 0.2	NP
pCD8	3	< 1	0 – 0.1	0 ^{††}
pCD29	3	95.57 \pm 1.36	92.9 – 97.4	98.1 \pm 1 [†]
pCD44	3	5.35	4.5, 6.2	60 \pm 15 [†]
hCD90	3	88.77 \pm 8.21	72.3 – 97.4	25.96*
pCD117	3	< 1	0.1 – 0.5	0.2 \pm 0.4 ^{††}
SSEA-4	3	1.7	0.2, 3.2	+**
mSca-1	3	< 1	0.2 – 0.3	NP

p = Porcine, h = Human, m = Mouse, NP = Not Published.

[‡]All human ASCs cells analyzed were early passages.

[†]Gronthos et al., J Cell Phys 2001; 189:54-63.

*Zuk et al., Mol Bio Cell 2002; 13:4279-4295.

^{††}Katz et al., Stem Cells 2005; 23:412-423.

**Lo Cicero et al., Cell Prolif 2008; 41:460-473.

Analysis of porcine ASCs at late PDs 10-34 also demonstrated that CD3, CD8, CD44, CD117, SSEA-4 and Sca-1 proteins tested were virtually non-existent on the surface of porcine ASCs (Table 4.2; Figure 4.1). However, CD29 expression remained elevated in the later PDs with a mean percent positive value of 7.80 ± 0.78 and a range of 6.5 – 9.2 (Table 4.2; Figure 4.1). Additionally, the percentage of positive events for CD90 (7.77 ± 3.83 ; range 0.3 – 13.0) remained elevated in the later passages. These values determined for CD29 and CD90 are more consistent with the values reported for the human ASCs, although it is important to note that the cells analyzed in these reports were early PDs and were not cultured as long as the cells in this study (Tables 4.1 and 4.2). Gronthos et al. studied cells cultured through two passages and cryopreserved (Gronthos et al., 2001), Katz et al. utilized human ASCs passage 2 or earlier (Katz et al., 2005) and Lo Cicero analyzed passage three cells (Lo Cicero et al., 2008). Nevertheless, the values are a good reference and our results indicate that the expression levels did not elevate in response to the duration in culture.

Western Blot Analysis of Stem Cell Surface Proteins

Immunoblot detection was paired with an ECL kit to confirm flow cytometric results of select stem cell surface markers analyzed. For both the human and porcine protein extractions, β -actin was utilized as a loading control and confirmed the presence of protein extraction in each sample (Figure 4.2). As expected, antibodies used against the human CD44 and CD90 proteins revealed a positive expression in human ASCs as indicated by bands present at the expected sizes (85 kDa and 25-35 kDa, respectively). However, the human antibodies did not detect expression for CD44 or CD90 in porcine ASCs by Western blot analysis (Figure 4.2). In response to this result we utilized a porcine antibody to CD44, but did not detect the protein in either the porcine or the human protein extractions (Figure 4.2). The porcine antibody to CD117 also did not identify the protein in the porcine or human protein extractions (Figure 4.2). The inability to detect any of the proteins analyzed by x-ray exposure verifies the findings derived by

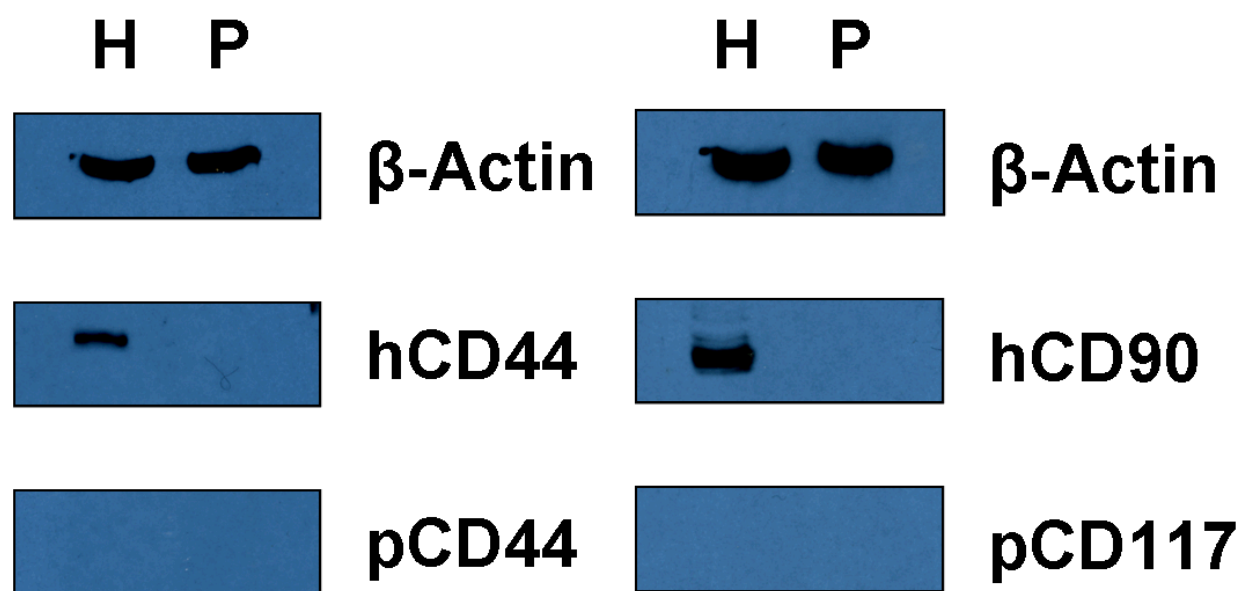


Figure 4.2. Western blot analysis of human and porcine ASCs for CD44, CD90 and CD117. Human CD44 and CD90 antibodies detected the protein in the human protein extraction (H) but not in the porcine protein extraction (P). Porcine CD44 and CD117 antibodies did not detect the protein in either the human or the porcine protein extractions.

flow cytometric analysis except for CD90. Again, we suspect that antibody cross reactivity did not occur between the pig in the human especially for the CD90 antigen.

Chromosomal Stability of pASC Through In Vitro Culture

Metaphase spreads were observed and selected following the previously described criteria. Digital images were captured for each metaphase spread and each chromosome was documented using a digital marking system. A normal porcine metaphase spread consisted of 38 chromosomes ($2n = 38$) (Figure 4.3 A) and an aneuploid cell contained chromosome numbers other than 38 ($38 \pm X$) (Figure 4.3 B). For each cell line, 20 to 30 metaphase spreads were analyzed per passage. Table 3 illustrates a total of 714 metaphase spreads analyzed for this study. Of 714 metaphases analyzed, 509 (71.3%) were aneuploid and only 205 (28.7%) were normal diploid porcine cells (Table 4.3). Hypoploid cells represented 66% (336) of aneuploid cells and the remaining 33.2% (169) (hyperploid and tetraploid combined) were hyperploid (Table 4.3). There were 153 (45.5%) cells that contained 37 or 36 chromosomes, 141 (42.0%) cells with a range of 35 to 32 chromosomes and 42 (12.5%) contained less than 32 chromosomes (Table 4.3).

For each cell line, (pASC 1, pASC 2 and pASC 3) we found a remarkable percentage of aneuploidies (43.7, 48.9 and 47.3, respectively, with a 46.6 ± 1.5 average) present immediately after the cultures were established (PD 1-10). Chi-square analysis indicates that the percents of aneuploid cells during PD 1-10 were significantly lower than those for PD 11-20 and PD 21-30 (Table 4.4) (Figure 4.4). The statistical difference was observed individually within a cell line and as an average of the three cell lines between PD groups (PD 1-10, PD 11-20 and PD 21-30) (Table 4.4; Figure 4.4).

Discussion

Previously, we determined that porcine ASCs expressed the classic characteristics of stem cells by demonstrating the ability to self-renew through ring cloning experiments and the ability to differentiate along multiple tissue lineages (Williams et al., 2008). Those findings were

A. Normal

Porcine Metaphase ($2n = 38$)



B. Aneuploid

Porcine Metaphase ($2n = 52$)

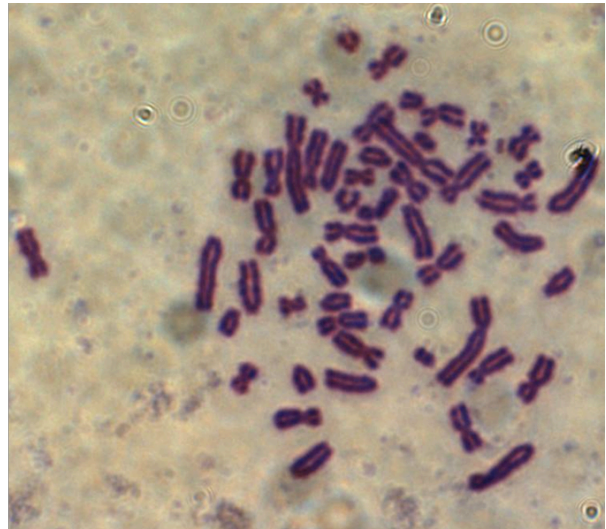


Figure 4.3. Metaphase spreads of porcine ASCs. Normal diploid porcine metaphase spread with 38 chromosomes (left). Aneuploid porcine metaphase spread with 52 chromosomes (right, magnification 100X oil immersion).

Table 4.3. Types of Aneuploidies in pASCs Derived By <i>In Vitro</i> Culture (PD 1-30)	
Total metaphases analyzed	714
Diploid cells (%)	205 (28.7)
Aneuploid cells (%)	509 (71.3)
Hypoploid cells = 38 – X (%)	336 (66.0)
37 – 36 chromosomes (%)	153 (45.5)
35 – 32 chromosomes (%)	141 (42.0)
< 32 chromosomes (%)	42 (12.5)
Hyperploid cells = 38 + X (%)	169 (33.2)
Tetraploid cells = 76 chromosomes (%)	4 (0.8)

Table 4.4. Chromosomal Stability of *In Vitro* Cultured pASC Population Through PD 30

Cell Line	N	Aneuploid Cells (%)		
		PD 1 – 10	PD 11 – 20	PD 21 – 30
pASC 1	243	43.7 ^a	87.1 ^b	96.8 ^b
pASC 2	220	48.9 ^a	82.1 ^b	100.0 ^b
pASC 3	251	47.3 ^a	91.7 ^b	86.3 ^b
Mean	714*	46.6 ± 1.5 ^a	87.0 ± 2.8 ^b	94.4 ± 4.1 ^b

n = number of metaphases analyzed per cell line.

*Total number of metaphases analyzed.

Standard error of the mean was calculated for the mean in each PD group.

Different letters within rows represent significant statistical differences between PD derived by Chi-square analysis ($P < 0.05$).

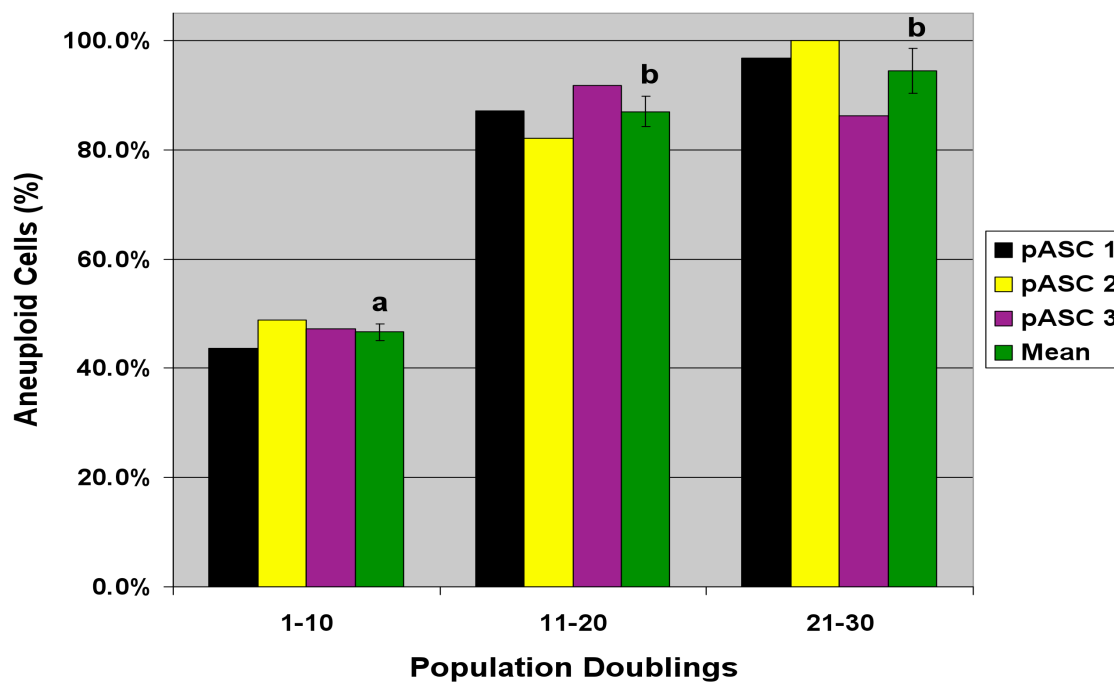


Figure 4.4. Chromosomal stability of porcine ASCs through *in vitro* culture. For pASC 1, pASC 2 and pASC 3 cells lines, the proportion of aneuploid cells was significantly higher in later PD (PD 11-20 and PD 21-30) than the earlier PD 1-10. Letters indicate significant differences between mean values of 46.6 ± 1.5 , 87.0 ± 2.8 and 94.4 ± 4.1 with a $P < 0.05$.

consistent with the characteristics determined in human ASCs (Halvorsen et al., 2001a; Hauner et al., 1989). Human and porcine adipose tissues offer an alternative stem cell source, can be accessed easily with little pain and are plentifully available. ASCs show much promise for use in reconstructive tissue engineering and therapeutic cloning. The present study is the first to describe the cell surface profile for stem cell associated surface markers comparing early and late passages of undifferentiated porcine ASCs as well as to determine the chromosomal stability through *in vitro* culture. The surface phenotype of porcine ASC is similar for some stem cell antigens and different for others when compared to human ASC cell surface as reported in the literature (Gronthos et al., 2001; Katz et al., 2005; Mitchell et al., 2006; Zuk et al., 2002).

Unlike reported values for human ASCs, porcine ASCs did not demonstrate positive expression for CD44 or SSEA-4. The absence of CD44 was confirmed by western blot analysis. However, our previous study did reveal positive gene expression of CD44 through reverse transcriptase polymerase chain reaction (Williams et al., 2008). Neither SSEA-4, found on bone marrow MSCs and human ASCs, nor Sca-1, also found on MSCs, was detected on the surface of porcine ASCs at early or late PDs. Consistent with the literature, however, CD29 and CD90 expression was detected in early and late PDs while CD8 and CD117 antigens were not present at early or late PDs. Our study is by no means a comprehensive investigation for stem cell markers potentially present on the surface of porcine ASCs. Bone marrow-derived MSCs and human ASCs have been more fully characterized in the literature and many more of these antigens need to be explored in porcine ASCs. Additional antigens for further investigation include CD13, CD49, CD51, CD61, CD63, CD73, CD105, CD106, CD138, CD140, CD146 and CD166 (Gronthos et al., 2001; Katz et al., 2005; Mitchell et al., 2006; Zuk et al., 2002). Cell surface proteins were chosen in the current study based on their availability and their indication of “stemness” in human ASC populations. At this time, the selection of antibodies commercially available is an obstacle for thoroughly investigating these characteristics in the porcine animal model. Human CD90 and mouse Sca-1 antibodies were used in flow cytometric analysis, while

human CD44 antibody (along with porcine CD44) was employed in the Western blot analysis. Antibody cross reactivity between species most likely did not occur as results indicate in the western blot analysis. However, all other antibodies were specific for porcine.

The inconsistency between porcine and human ASC surface may be due to several factors effecting the *in vitro* culture environment. It has been proposed that the expression pattern may not be reproducible because of cellular response to a hostile environment (Katz et al., 2005). The exposure of cells to culture vessel plastic, the specific medium used, the duration of culture (i.e. passage number, cell cycle length), the seeding density or whether the cells have been cryopreserved prior to culture all could influence the phenotype of the cell as it adapts to this environment (Katz et al., 2005; Planat-Benard et al., 2004; Rehman et al., 2004). Another important factor is the tissue donor including the age of the donor and the physiological location of tissue collection. Human ASCs are derived from processed liposuction aspirates where in this study porcine ASCs were harvested from subcutaneous biopsies.

The age of the donor dictates the life expectancy of the cells in culture (Cristofalo et al., 1998). This phenomenon is extremely important when selecting cells to be used as donor cells in NT. The process of NT requires a donor cell with a normal diploid that has not yet reached senescence. The establishment of an applicable genetically modified donor cell requires multiple PDs, which increases the probability that the cells will acquire cytokinetic abnormalities and chromosomal alterations. The use of an afflicted donor cell is most likely responsible for the production of abnormal embryos that do not survive and may be the culprit of extremely low success rates in NT.

Studies of chromosomal integrity through *in vitro* culture are quite limited at this time. Giraldo et al. in 2006 characterized the proliferative capacity and the chromosomal stability of bovine fetal fibroblasts. The level of aneuploid cells was highly elevated with a mean value of 47.9 ± 5.5 immediately after the primary cultures were established (Giraldo et al., 2006). As the cells progressed through culture the aneuploid levels dramatically increased (67.4 ± 1.4 for

middle PDs and 85.0 ± 4.3 for late PDs (Giraldo et al., 2006). Our findings for porcine ASCs were very similar. Porcine ASCs demonstrated an increased percentage of aneuploid cells directly from the initiation of culture with a mean value of 46.6 ± 1.5 . The percentage of aneuploid cells significantly increased for middle PDs (87.0 ± 2.8) and late PDs (94.4 ± 4.1). We conclude that the assault to chromosomal stability clearly occurs after removing the cells from their *in vivo* environment, which is supported by such elevated levels of aneuploidy directly from the start in both bovine fetal fibroblasts and porcine ASCs. The suboptimal culture conditions continue to corrode the genetic stability of the cells as they fight to survive *in vitro* culture through middle and late PDs as indicated by the significant increases in aneuploidy.

NT efficiency may significantly increase with the implementation of a method to select karyotypically normal diploid cells since approximately 50% of *in vitro*-cultured cells in early PDs are aneuploid. However, the techniques available at this time for determining chromosomal content are terminally destructive to the cell. Until new methodology can be discovered, it is advantageous to choose donor cells at younger PDs. With this in mind, improving the methods for generating transfected cells for NT at younger PDs would also likely increase the efficiency of NT techniques.

The availability of an animal model for somatic stem cells will aid in tissue regeneration, gene therapies and the development of other stem cell research techniques. The differences found between porcine ASC and human ASC surface markers may be attributed to many factors including the location of the fat depot as well as the age of the donor. Therefore care should be taken when interpreting results from animal models of somatic stem cells. A selection procedure for younger NT donor cells will minimize chromosomal defects and increase reprogrammability necessary for healthy embryo production. Continued research will be needed to identify a selection method as well as improve the manipulation of other cellular mechanisms involved in the process of NT.

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CHAPTER V

EPIGENETIC MODIFICATIONS OF CULTURED PORCINE ADIPOSE TISSUE-DERIVED STEM CELLS

Introduction

Currently, nuclear transfer results in relatively low success rates and investigators have reported substantial problems with the welfare of the cloned animals. Developmental abnormalities observed following nuclear transfer define Large Offspring Syndrome or LOS and include low pregnancy rates, high percentages of loss during early and late pregnancy, stillbirths, early postnatal deaths, short life-span, obesity and malformations. LOS may be caused by several factors such as using an inappropriate donor cell and/or recipient oocyte, the inability to properly synchronize the cell cycle phase of the donor nucleus and the recipient cytoplasm and inadequate reprogramming of the donor genome. Also, the inappropriate handling of oocytes, somatic cells and embryos during maturation with the subjection of these cells to the various necessary manipulations and culture techniques could result in cellular stresses leading to LOS.

A shift in research has focused on the epigenetic modifications of DNA and their role in embryonic development. In the production of a normal developing embryo, the oocyte (arrested at metaphase II) is united with a sperm and completes meiosis and extrudes the second polar body. The embryo contains a subset of cells that remain totipotent (can support differentiation into all cell types) for the first one to two cell divisions (Kono, 1997). The following cleavage events undergo DNA modifications, which render them unable to form all tissue types and once initiated it is believed that the series of events proceeds beyond the point of no return. However, exposure of a donor cell's chromatin to ooplasm as in nuclear transfer will redirect the programming of the donor nucleus to mimic that of a zygotic nucleus as in normal fertilization (Kikyo and Wolffe, 2000). The oocyte virtually erases the epigenetic modifications imposed on the genome during differentiation and reinstates totipotency (Kikyo and Wolffe, 2000). The

reprogramming of the donor cell nucleus is essential to initiate and complete embryonic development.

Fusion with embryonic stem cells provides an alternative method for inducing nuclear reprogramming of somatic nuclei. Factors within the embryonic stem cell, much like in the unfertilized oocyte, render a resultant hybrid cell, which demonstrates pluripotent competence by differentiation into multiple cell lineages and expression of tissue-specific genes (Cowan et al., 2005; Tada et al., 2001). Several somatic cell types have been successfully reprogrammed upon fusion with embryonic stem cells including thymocytes (Tada et al., 2001), human fibroblasts (Cowan et al., 2005), and human myeloid precursor cells (Yu et al., 2006). Research indicates that the nuclear compartment of the embryonic stem cell houses the reprogramming capability because fusion of karyoplasts and not cytoplasts generate the reprogrammed hybrid (Do and Scholer, 2004).

These reprogrammable epigenetics govern the regulation of repression of genes not necessary in cell types at specific developmental stages while preserving the DNA sequence (Wolffe and Matzke, 1999). Epigenetic modifications during the reprogramming process following nuclear transfer include DNA methylation and posttranslational acetylation, phosphorylation and methylation of histone N termini that function to modulate chromatin structure, which ultimately regulates transcriptional activation or repression of specific genes. Acetylable lysine residues in the N termini of histone H3 (such as K9, K14, K18, and K23) and H4 (such as K5, K8, K12, and K16) and methylable lysine residues in H3 (such as K4, K9, K27, K36, and K79) and H4 (K20) are only a few that have been identified (Kimura et al., 2004). Histone acetyltransferases (HATs) are the enzymes responsible for the transfer of acetyl groups to lysine residues and histone deacetylases (HDACs) dictate their removal. Acetylation neutralizes the positive amino acid charge decreasing the affinity for DNA. The termini displace from the nucleosome increasing accessibility of transcription factors, which leads to gene expression (Grunstein, 1997). Alternatively, DNA methyltransferases (Dnmts) coordinate

methyl group addition to DNA, which demarcates or “imprints” portions of DNA in order to distinguish between silenced and expressed genes of parental alleles (Cezar, 2003).

H19, insulin-like growth factor II, and the insulin-like growth factor II receptor are examples of genes that display alternative DNA methylation patterns based on the inheritance of the allele either from the sperm or the oocyte (Kono, 1997). Generally, DNA methylation blocks transcription factors from binding the methylated sequences and is associated with transcriptional silencing and imprinting (Hiendleder et al., 2004b). The transcription repressor protein, methyl CpG-binding protein-2 (MeCP2), binds methylated DNA which then as a complex recruits HDACs (Jones et al., 1998; Nan et al., 1998). The removal of acetyl groups from histone tails increases histone affinity for DNA further preventing transcription factor accessibility (Cezar, 2003).

In nuclear transfer, genes normally expressed during embryogenesis are silent in somatic donor cells and must be turned back on. Therefore, the DNA methylation patterns must be reorganized to resemble that of undifferentiated nuclei (Rideout et al., 2001). LOS and other phenotypic abnormalities may be a result of improper expression of imprinted genes as a result of reorganized DNA methylation (Kono, 1997).

Regulators of development in undifferentiated cells are suggested to be silenced by the presence of a bivalent domain modification pattern in which a large region of histone 3 lysine 27 trimethylation (H3K27me3) contains smaller regions of histone 3 lysine 4 trimethylation (H3K4me3) (Bernstein et al., 2006). Important to the maintenance and pluripotency of stem cells, Polycomb-group proteins catalyze the H3K27me3 modification and Trithorax group proteins catalyze the H3K4me3 modification (Bernstein et al., 2006). The dual marks contain both repressive (H3K27me3) and activating (H3K4me3) modifications, which work to silence developmental genes in embryonic stem cells while simultaneously keeping them receptive to activation. As the cell differentiates, the bivalent conformation resolves where H3K4me3 activating marks permit early-replicating gene expression and H3K27me3 repressing marks

block late-replicating gene expression in cells committed to a specific lineage (Boyer et al., 2006; Meshorer and Misteli, 2006).

Also of note, these developmental regulators contain highly conserved noncoding elements, which contain a wealth of H3K27me3 and H3K4me3 harboring bivalent domains (Bernstein et al., 2006). These regions house genes responsible for encoding several transcription factors that function to maintain and regulate pluripotency and self-renewal in both early embryos and embryonic stem cells (Bernstein et al., 2006). These factors include the POU (Pit/Oct/Unc) domain-containing protein Oct-4 (also known as Pou5f1) (Nichols et al., 1998; Niwa et al., 2000; Rosner et al., 1990; Scholer et al., 1990a; Scholer et al., 1990b), the high mobility group (HMG)-box protein Sox-2 (Avilion et al., 2003) and the homeoprotein Nanog (Chambers et al., 2003; Mitsui et al., 2003). These three transcription factors function through very distinct pathways that are thought to converge to dictate early cell fate decisions (Boyer et al., 2006). Recently, the conservation of these proteins among mammalian species has become apparent and Oct-4, Sox-2 and Nanog are considered markers of cells that have pluripotent capabilities. Also, their association with highly conserved non-coding elements suggests a conserved role for the bivalent chromatin domains mentioned above (Bernstein et al., 2006).

The initial epigenetic landscape of the DNA sequence determines the pluripotent capabilities of the cell. Embryonic stem cells possess transcriptionally active chromatin that is structured in an open configuration containing an abundance of acetylated lysine residues (Boyer et al., 2006). However, as the cell commits to a differentiation pathway, acetylated histone modifications decrease, heterochromatin formation ensues and pluripotential declines (Meshorer and Misteli, 2006; Meshorer et al., 2006).

At this time, research has not identified epigenetic modifications in adult somatic MSCs from porcine adipose tissue. Analysis of these cells may reveal global methylation and/or acetylation patterns similar to their embryonic stem cell counterparts. Further characterization

of porcine ASCs in this manner could assist the isolation and purification of a population of MSCs from the easily obtainable tissue of adipose that can later be tested as donor cells in nuclear transfer. Somatic cells used as donor cells in nuclear transfer typically produce more blastocyst stage embryos compared to embryos derived from embryonic stem cells (Rideout et al., 2001). Approximately 60% of embryonic stem cells are in S-phase when used as donor cells (Rideout et al., 2001). S-phase embryonic stem cells are exposed to high levels of maturation promoting factor, which induces premature nuclear envelope breakdown and chromosome condensation. Cells likely undergo a second DNA replication cycle activating cell checkpoint mechanisms ultimately leading to inappropriate ploidy and cell cycle arrest. However, more embryonic stem cell nuclear transfer embryos develop to term than somatic cell nuclear transfer embryos (Rideout et al., 2001). Although nuclear transfer clones produced from either embryonic stem cells or somatic cells demonstrate similar phenotypic abnormalities, term embryonic stem cell nuclear transfer clones indicate that utilization of a less differentiated donor cell may be more acquiescent to appropriate reprogramming (Rideout et al., 2001).

Our overarching hypothesis is that low development rates observed following porcine nuclear transfer are a result of incomplete reprogramming of the donor cell nucleus and that somatic stem cells or those cells which demonstrate stem-like characteristics (*i.e.*, ASCs) will be reprogrammed more completely. New information concerning the nature of the epigenetic landscape will aid the efficiency of isolation and purification of a population of MSCs from the easily obtainable tissue of porcine adipose that can later be tested as donor cells in nuclear transfer.

Materials and Methods

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Establishment of Primary Cultures and Cell Culture Maintenance

Primary cultures were established following procedures previously documented (Williams et al., 2008). Briefly, 10 g subcutaneous fat was harvested from the dorsal abdominal area in mature female cross-bred domestic pigs between the ages of 6 months to 1 year at a local abattoir. The tissue was finely minced and washed with Dulbecco's phosphate-buffered saline (dPBS) containing 200 U/ml of penicillin and 200 µg/ml of streptomycin and 2.5 µg/ml of Fungizone (Gibco). Each sample was homogenized with an equal volume of 0.1% collagenase Type I (240 IU/ml; Gibco) in dPBS with 1% bovine serum albumin (BSA) in a shaker incubator rotating at 200 rpm at 39°C for 2 h.

The cell suspension was filtered through a dual filter system comprised of two nylon net filters (20 µm and 180 µm) (Millipore, Bedford, MA, USA). Cells were then stained with Hoechst 33342 (2 µg/ml) to determine the number of nucleated cells in the suspension. Cells were plated at 6,700 cells/cm² in 10 ml of expansion medium containing Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, Logan, UT, USA) with high glucose supplemented with 10% fetal bovine serum (FBS) (Hyclone), 100 U/ml of penicillin and 100 µg/ml of streptomycin and 2.5 µg/ml of Fungizone in a 150 cm² culture flask and incubated at 39°C in 5% CO₂.

Fresh medium was supplied every 3 to 4 days. When 70 to 90% confluence was achieved, the porcine adipose-derived stem cells (pASCs) were harvested by trypsinization, counted using a hemacytometer and re-seeded at an initial concentration of 6,700 cells/cm². Cells were passaged until replicative senescence was noted.

Differentiation of pASC Cultures

At 70 to 90% confluence (following passage 6), cells were passaged by trypsinization and cultured for 21 days in culture medium previously demonstrated to induce differentiation (Zuk et al., 2002; Gimble and Guilak, 2003). Culture media were changed every 3 to 4 days throughout the study. For adipogenesis, cells were cultured on days 1 to 4 in differentiation medium containing DMEM supplemented with 3% FBS, 100 U/ml of penicillin and 100 µg/ml of

streptomycin, 2.5 µg/ml of Fungizone, 10 ng/ml of insulin, 10^{-9} M dexamethasone, 250 µM isobutyl methylxanthine and 0.2 mM indomethacin. Culture medium beyond day 4 contained DMEM with 3% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin, 2.5 µg/ml of Fungizone, 10 ng/ml of insulin and 10^{-9} M dexamethasone. For chondrogenesis, cells were cultured for the duration of differentiation in medium containing DMEM with 10% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin, 2.5 µg/ml of Fungizone, 6.25 µg/ml of insulin, 10 ng/ml of transforming growth factor- β and 10^{-9} M dexamethasone. For osteogenesis, cells were cultured in differentiation medium containing DMEM with 10% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin, 2.5 µg/ml of Fungizone, 10 mM β -glycerophosphate, 50 ng/ml of ascorbic acid and 10^{-9} M dexamethasone.

Histological staining techniques were used to determine differentiation. Nile Red, a stain, which detects intracellular lipid accumulation, was used to determine adipocyte differentiation. Safranin O was utilized to identify glycosaminoglycans in the extracellular matrix of differentiated chondrocytes. After osteogenic induction, Alizarin Red S was employed to detect mineralized calcium deposition.

Stem Cell Surface Immunolabeling for Methylated DNA and Acetylated Histone H3

Porcine ASCs were harvested by trypsinization, washed and resuspended in dPBS. The protocol established by Habib et al. (1999) was used to fix and immunolabel cells. Briefly, pASCs were permeated by 3 washes in a dPBS solution containing 1% BSA and 0.1% Tween-20 (PBST-BSA). pASCs were then fixed in a 0.25% paraformaldehyde solution for 10 min at 37°C and then stored in 90% cold methanol at -20°C at least overnight. Following two washes in PBST-BSA, cells were treated with 2N HCl for 30 minutes at 37°C. A 0.1M boric acid buffer (pH 8.5) was used to neutralize the fixative for 5 minutes at room temperature. pASCs were washed 3 times in a dPBS solution containing 1% BSA and incubated in the last wash for 30 minutes at room temperature to block nonspecific binding sites. For methylated DNA, pASCs were incubated first in dPBS with 5 µg/ml mouse anti-5-methylcytidine (AbDSerotec, Oxford,

UK; Catalog # MCA2201) for 30 min at room temperature followed by incubation in dPBS with 20 µg/ml Alexa Flour® 488 goat anti-mouse IgG (H+L) (Carlsbad, CA, USA; Catalog # A11001) for 30 minutes at room temperature. For acetylated histone H3, pASCs were incubated first in dPBS with 10 µg/ml anti-acetyl-Histone H3 (rabbit polyclonal IgG) (Millipore, Temecula, CA; Catalog # 06-599) for 30 minutes at room temperature followed by incubation in dPBS with 20 µg/ml Alexa Flour® 488 goat anti-rabbit IgG (H+L) (Carlsbad, CA, USA; Catalog # A11008) for 30 min at room temperature. pASCs were then counterstained in a 0.112% sodium citrate solution containing 50 µg/ml propidium iodide and 10 µg/ml RNase for 30 min at room temperature. pASCs were washed and resuspended in dPBS at a concentration of approximately 1×10^6 pASCs/ml for flow cytometric analysis. A portion of cells was observed under 100x magnification to verify antibody specificity for nuclear localization (Figure 5.3).

Flow Cytometric Analysis of Epigenetic Modifications

Samples were analyzed for relative levels of methylated DNA and acetylated histone H3 using the Accuri™ C6 Flow Cytometer® (Accuri Cytometers, Ann Arbor, MI, USA). At each passage (1, 2, 3, 4, 5, 7 and 9) for every line analyzed (FPF 1, 2, 3 and 4, and pASC 1, 2, 3 and 4), at least 10,000 cumulative events were collected. Labeled cells were excited with a 488 nm diode laser and a >670 nm band pass filter was used to detect PI levels (FL3-channel). The DNA content derived from the PI levels was used to gate cells in G0/G1 phase (FL3-channel). Total methylated DNA and acetylated histone H3 levels in G0/G1 cells were then detected in the FL1-channel at 530/30 nm. Dot plots and histograms were derived using the Accuri CFlow Plus® (Accuri Cytometers, Ann Arbor, MI, USA) software. Positive fluorescent events detected in the FL1-channel were designated a mean value generated by the software and recorded as arbitrary fluorescence units (AFU).

RNA Extraction and RT-PCR for Chromatin Remodeling Proteins

Total pASC and fetal porcine fibroblast (FPF) messenger RNA was isolated from approximately 1×10^6 cells using the TRI-Reagent® RNA/DNA/Protein Isolation Reagent

(Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. Briefly, media was discarded from cultured pASCs and FPFs grown in a monolayer. The TRI reagent was added to monolayers and allowed to incubate at room temperature for 5 minutes. Cell lysates were passed several times through a pipette and transferred to 15 ml phase lock tubes. The BCP reagent (1-bromo-3 chloropropane) was then added to the lysates, shaken vigorously and incubated at room temperature for 1 hour. Lysates were centrifuged at 12,000 x g for 15 minutes at 4°C to separate phases in which the upper transparent aqueous phase was transferred to a clean 15 ml centrifuge tube. Isopropanol was added to the aqueous phase and shaken to extract the RNA. Samples were stored at -20°C overnight and centrifuged at 12,000 x g for 20 minutes at 4°C the following day. The RNA pellet was then washed with 75% ethanol made with DEPC treated water, air dried and then resuspended in DEPC treated water. Extracted total RNA was stored in -20°C for approximately 3 months and then reverse transcribed using the iScript® cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. The iScript® reverse transcription (RT) reaction volume was carried out in 20 µl total and contained 4 µl of iScript® reaction mix, 15 µl of mRNA and 1 µl reverse transcriptase. Reaction mixes were concocted for each sample and a no reverse transcriptase negative control. Negative control reactions were analyzed by gel electrophoresis to verify the absence of contaminating genomic DNA (data not shown).

Quantitative Real Time PCR for Chromatin Remodeling Proteins

PCR primers were designed using the Beacon Designer 4.0 (PREMIER Biosoft International, Palo Alto, CA, USA) software for the amplification of DNA methyltransferase 1 (Dnmt1), Dnmt3a, histone deacetylase 1 (HDAC1), methyl CpG-binding protein-2 (MeCP2) and the reference gene polyadenylate polymerase (Poly A) from both porcine and bovine gene sequences (Table 5.1). cDNA was amplified in a reaction mix with a total volume of 25 µl

Table 5.1. Oligonucleotide PCR primers for real time quantitative PCR

Gene	Forward/Reverse	Accession Number	Primer	Amplicon length (bp)
Gene Expression Analysis of Chromatin Remodeling Proteins				
pDnmt1	F	NM_001032355	CAGACAAGTGGTGAGGAC	186
	R		TGTGAAATGAGATGTGATGG	
pDnmt3a	F	DQ785811	CCCGAAAGAGCACAACCTG	119
	R		GCACATTCCTCCGATGAAG	
pHDAC1	F	AK230658	CATCTGGCTTCTGTTACG	139
	R		ACTCGGTCTGTGGTATAG	
bMeCP2	F	XM_588477	CCGCTGGGAAGTATGATGTG	191
	R		GGGAGATTTGGGCTTCTTAGG	
pPolyA	F	AK233143	GACTGACTGCCTGCTTACAC	137
	R		TTTCTCGTATCCACTCTTTGACC	
Chromatin Immunoprecipitation Analysis of Pluripotent Transcription Factors				
pOCT4 Proximal	F	NC_010449	CAGAGGTCAAGGCTAGTG	214
	R		CCAGGTCCGAGGATCAAC	
pOCT4 Distal	F	NC_010449	GGTGTCCGATTGATTCCTC	200
	R		GCCCAGCCTATTAGATTCC	
pNANOG Proximal*	F	EF522119	AATTCGCAAGGGTGTGTGTG	128
	R		CAGGAGCTAACCACCCCAAT	
pNANOG Distal*	F	EF522119	AGCTCATGGCAACGCTAGAT	115
	R		TGACAGGAGTTTCCATCGTG	

p = porcine; b = bovine; Dnmt = DNA methyltransferase; HDAC = histone deacetylase; MeCP = methyl CpG-binding protein; PolyA = polyadenylate polymerase.

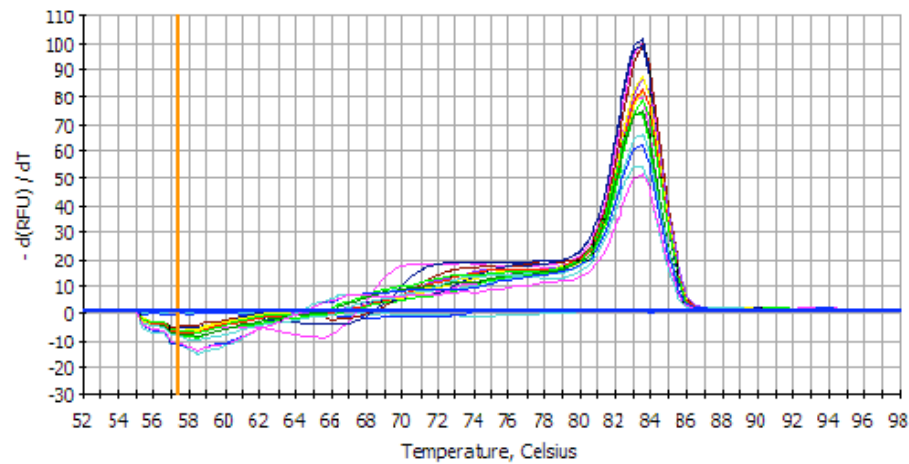
*Miyamoto et al., (2009).

containing 12.5 μ l iQTMSYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 2 μ l cDNA, 8.5 μ l nuclease-free water and 1 μ l each of sense and antisense primers (20 pmol) for each gene. Reaction mixes were concocted for each sample, a calibrator (derived from porcine muscle tissue) and a no reverse transcriptase negative control. Quantitative real time PCR (qPCR) was performed for all genes including the reference gene Poly A in the MyiQ Reverse Transcription PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the program consisting of a denaturing cycle of 3 minutes at 95°C, 40 cycles of PCR (10 seconds at 95°C, 45 seconds at 55°C and 1 minute at 95°C), a melting curve for 1 minute at 95°C followed by 1 minute at 55°C, a step cycle starting at 55°C for 10 seconds with a 0.5°C/second transition rate, and a constant cooling cycle held at 4°C.

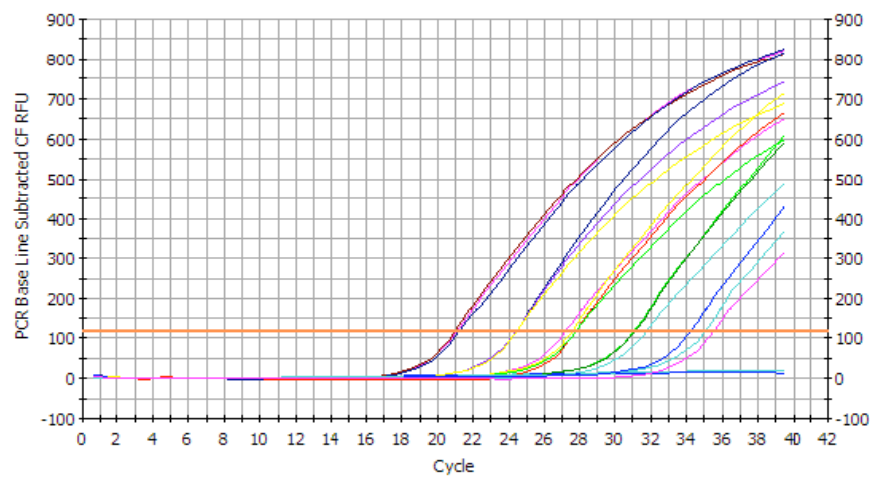
Livak and Schmittgen describe the comparative quantification cycle (C_q) method used to analyze qPCR data (Livak and Schmittgen, 2001). The data is reported as an n-fold change in gene expression relative to the calibrator. The C_q of the reference gene Poly A was used to normalize the target gene C_q in each sample. The ΔC_q for the transcription of each gene was then calibrated against cells from porcine muscle tissue. The mean fold change in expression of the target gene relative to the calibrator was calculated by $2^{-\Delta\Delta C_q}$ and presented as fold change in target gene expression.

All primers were validated for annealing temperature by performing an annealing temperature gradient and subsequent analysis by gel electrophoresis (data not shown). Also, primers were validated for the amplification of a single product in a quantitative manner, efficiency level, correlation coefficient and melting curves of cDNA at five different dilutions using qPCR (Figure 5.1). All target genes analyzed generated efficiencies between 95 to 113% and correlation coefficients between 0.99 to 1.00.

A.



B.



C.

Correlation Coefficient: 0.998 Slope: -3.455 Intercept: 20.997 $Y = -3.455X + 20.997$
PCR Efficiency: 94.7 %

Unknowns
Standards

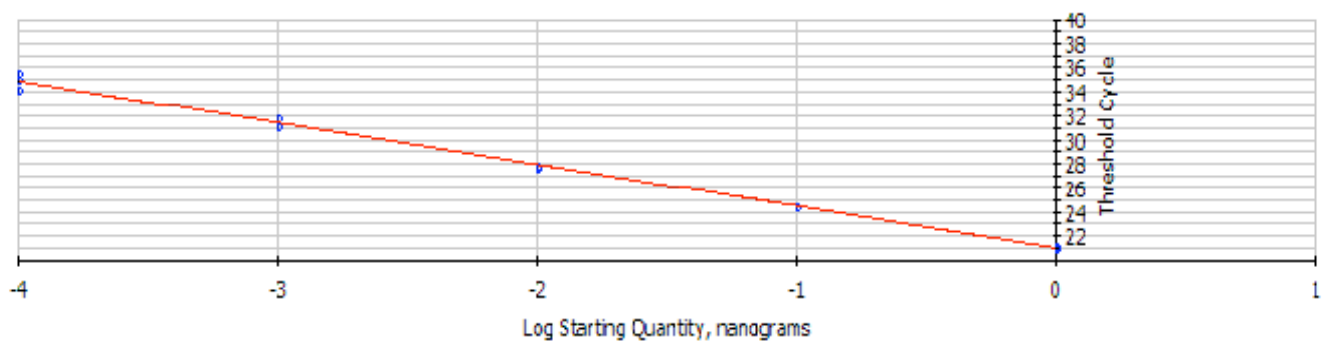


Figure 5.1 Primer validation. Representative melt curve (A), threshold cycle (Ct) values (B) and standard curve (C) derived from a 1:10 template dilution series for the validation of forward and reverse primers.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were carried out according to the protocol described by Dahl and Collas (Dahl and Collas, 2008) with a few modifications. Cells were harvested by trypsinization as per standard protocol described above. Cells were counted using a hemacytometer and approximately 2.5×10^6 cells were allocated to the ChIP assay. The use of sodium butyrate in all solutions and buffers throughout the protocol was eliminated on the basis that the epigenetic modification under investigation was not histone acetylation. Cells were resuspended in 500 μ l PBS. DNA-protein crosslinking was performed by paraformaldehyde (1% v/v), vortexed gently and incubated at room temperature for 8 minutes. The reaction was terminated with the addition of glycine (125 mM final concentration) and incubated at room temperature for 5 minutes.

Sonication of chromatin was performed on a Branson Sonifier 250 Duty cycle 40% Level 4 for 10 times 20 pulses on ice with a 1 minute pause in between each set of 20 pulses. Preparation of antibody-bead complexes was performed by incubating 11 μ l well-suspended protein A bead stock from Millipore MagnaChIP kit (Millipore, Billerica, MA, USA, Catalog #MAGNA001) with 2.4 μ g of antibody at 4°C for 2 hours at 40 rpm in a hybridization oven. Chromatin was immunoprecipitated by incubating 100 μ l diluted chromatin with the antibody-bead complexes at 4°C overnight at 40 rpm in a hybridization oven. The DNA was eluted per Dahl and Collas protocol and isolated using the spin filter columns provided in the Millipore MagnaChIP kit following the manufacturer's instructions.

PCR primers were designed using the Beacon Designer 4.0 (PREMIER Biosoft International, Palo Alto, CA, USA) software for the amplification of a proximal and distal region from the initiation of transcription within the promoter regions of both Oct-4 and Nanog (Table 5.1). cDNA was amplified in a reaction mix with a total volume of 25 μ l containing 12.5 μ l iQTMSYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 2 μ l cDNA, 8.5 μ l nuclease-free water and 1 μ l each of sense and antisense primers (20 pmol) for each gene.

Reaction mixes were concocted for each sample, a calibrator (derived from porcine muscle tissue) and a no reverse transcriptase negative control. Quantitative real time PCR (qPCR) was performed for all genes including the reference gene Poly A in the MyiQ Reverse Transcription PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the program consisting of a denaturing cycle of 3 minutes at 95°C, 40 cycles of PCR (10 seconds at 95°C, 45 seconds at 55°C and 1 minute at 95°C), a melting curve for 1 minute at 95°C followed by 1 minute at 55°C, a step cycle starting at 55°C for 10 seconds with a 0.5°C/second transition rate, and a constant cooling cycle held at 4°C.

We present the data as fold enrichment of precipitated DNA associated with either H3K4me3 or H3K27me3 relative to a 1/100 dilution of input chromatin representing the percentage of precipitate material relative to the amount in the input sample.

Experimental Design

Four cell lines each were established for porcine ASCs and porcine FPFs. Cells were cultured until senescence was reached. At each passage analyzed, cells were trypsinized and re-seeded at 30 to 40% confluence to avoid possible contact inhibition and differentiation. Passaged lines were cultured until 70 to 90% confluence was reached. Following passage 6, a portion of pASC cell lines were cultured under differentiation conditions to assess the stem cell characteristics following that duration through *in vitro* culture. Relative levels of methylated DNA and acetylated histone H3 as well as levels of chromatin remodeling protein expression were analyzed for both porcine ASCs and porcine FPFs at each passage. These data are reported for each passage analyzed, however, the population doublings at each passage for each respective cell type is different based on their growth characteristics (PD 3, 5, 7, 10, 12 and 16 respectively for FPFs and PD 1, 3, 5, 7, 8, 10 and 11, respectively, for pASCs).

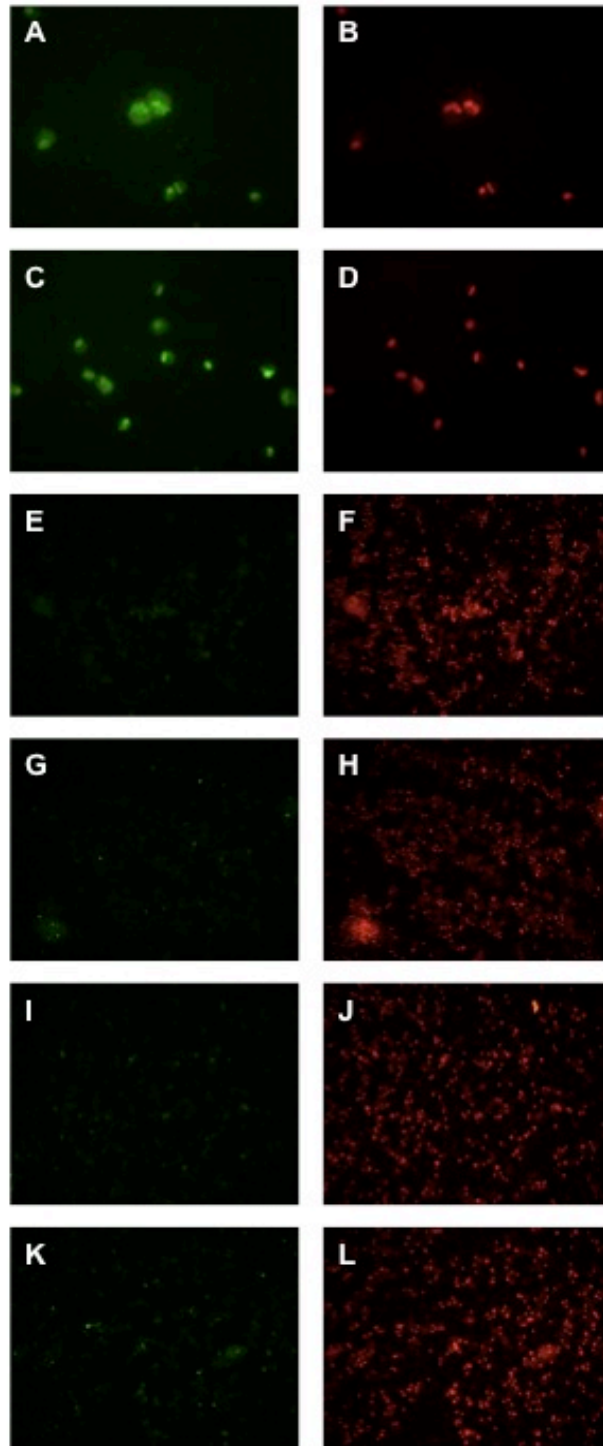


Figure 5.2. Porcine fibroblasts and pASCs stained with anti-5-methylcytidine and anti-acetyl-histone H3. pASCs stained with anti-acetyl-histone H3 (A and E), counterstained with PI (B and F), anti-5-methylcytidine (C and G) and counterstained with PI (D and H). FPFs stained with anti-acetyl-histone H3 (I), counterstained with PI (J), anti-5-methylcytidine (K) and counterstained with PI (L).

Statistical Analysis

In this study, chi-square analysis was employed to karyotypic data in order to determine statistical differences between cell lines (pASC 1, pASC 2 and pASC 3) and PD (PD 1-10, PD 11-20 and PD 21-30). For this test, a $P < 0.05$ was considered significant. Data were analyzed using the SigmaStat Statistical Software Version 3.5 (Systat Software, Inc., San Jose, CA, USA). For DNA methylation and histone acetylation, student's t-test was used to determine statistical differences between cell types using a P value of < 0.05 . Also, Tukey's test was used to determine statistical differences between passages using a P value of < 0.05 . For chromatin remodeling protein gene expression data, the Proc Mixed with repeated measures procedure was used in SAS statistical software to determine statistical differences between cell type and passages.

Results

Differentiation of pASCs

Following passage 6, a portion of pASCs was cultured in differentiation conditions for adipogenesis, chondrogenesis and osteogenesis. All four pASC cell lines successfully differentiated under adipogenic, chondrogenic and osteogenic conditions (data not shown). Cells cultured under adipogenic conditions stained positive for intracellular lipids. Chondrogenic induction generated cells that stained positive for the presence of glycosaminoglycans. Lastly, osteogenic differentiation was detected by the presence of calcium deposition in cultured cells.

Antibody Specificity for Methylated DNA and Acetylated Histone H3

Epifluorescence microscopy reveals the specificity of high intensity staining for anti-acetyl-histone H3 and anti-5-methylcytidine to the nuclear compartment for pASCs (Figure 5.2 A and C, respectively). Counterstaining with PI confirmed the site of the fluorescent staining by corresponding to DNA detected within the nucleus (Figure 5.2 B and D). A degree of cytoplasmic fluorescence can be observed in both pASCs (Figure 5.2 A, C, E and G) and FPFs (Figure 5.2 I and K), which may contribute to background fluorescence.

Table 5.2. Levels of Methylated DNA in FFs and ASCs through *in vitro* culture

Cell Line	Methylated DNA (AFU) ± SEM						P9
	P1	P2	P3	P4	P5	P7	
FPF 1	3990	2700	3106	2480	3264	2744	
FPF 2	6510	5782	3984	5214	5993	2972	
FPF 3	6691	4724	3691	3350	3031	2946	
FPF 4	7337	4360	4213	5100	5515	3241	
Mean	6132 ± 736 ^{a,1}	4391 ± 639 ^{a,1,2}	3749 ± 239 ^{a,1,2}	4036 ± 671 ^{a,1,2}	4451 ± 760 ^{a,1,2}	2976 ± 102 ^{a,2}	
pASC 1	8856	1836	1110	1001	1834	1965	6187
pASC 2	5236	1864	1034	1044	1599	2506	9336
pASC 3	8941	1346	1692	1422	2069	1961	4003
pASC 4	4901	2210	1342	1152	1675	1551	101
Mean	6984 ± 1108 ^{a,1}	1814 ± 178 ^{b,1,2}	1294 ± 148 ^{b,2}	1155 ± 94 ^{b,2}	1794 ± 104 ^{b,1,2}	1996 ± 196 ^{b,1,2}	4907 ± 1940 ^{1,2}

AFU, arbitrary fluorescence units; SEM, standard error of the mean.

Different letters (a and b) within columns indicate significant difference between cell types.

Different numbers (1 and 2) within rows indicate significant difference between passages.

Student's t-test was used to determine statistical differences between cell types ($P < 0.05$).

Tukey's test was used to determine statistical differences between passages ($P < 0.05$).

Table 5.3. Levels of Acetylated Histone H3 in FFs and ASCs through *in vitro* culture

Cell Line	Acetylated Histone H3 (AFU) \pm SEM						P9
	P1	P2	P3	P4	P5	P7	
FPF 1	7392	7809	8644	3369	4269	3261	
FPF 2	7597	9232	7384	4142	4646	3955	
FPF 3	8654	6654	7108	1372	4526	2931	
FPF 4	9029	6489	6330	3419	4039	3099	
Mean	8168 \pm 399 ^{a,1}	7546 \pm 634 ^{a,1}	7366 \pm 481 ^{a,1}	3076 \pm 595 ^{a,2}	4370 \pm 136 ^{a,2}	3312 \pm 225 ^{a,2}	
pASC 1	17145	11654	4321	3173	4516	3124	12090
pASC 2	10248	8997	5571	3063	3668	3061	13725
pASC 3	9811	7150	5377	2793	3558	3284	5883
pASC 4	8126	7536	5170	2621	3270	2108	5017
Mean	11333 \pm 1991 ^{a,1,2}	8834 \pm 1020 ^{a,1,2}	5109 \pm 275 ^{b,1,2,3}	2913 \pm 126 ^{a,3}	3753 \pm 268 ^{a,1,2,3}	2894 \pm 266 ^{a,2,3}	9179 \pm 2186 ^{1,2,3}

AFU, arbitrary fluorescence units; SEM, standard error of the mean.

Different letters (a and b) within columns indicate significant difference between cell types.

Different numbers (1, 2 and 3) within rows indicate significant difference between passages.

Student's t-test was used to determine statistical differences between cell types ($P < 0.05$).

Tukey's test was used to determine statistical differences between passages ($P < 0.05$).

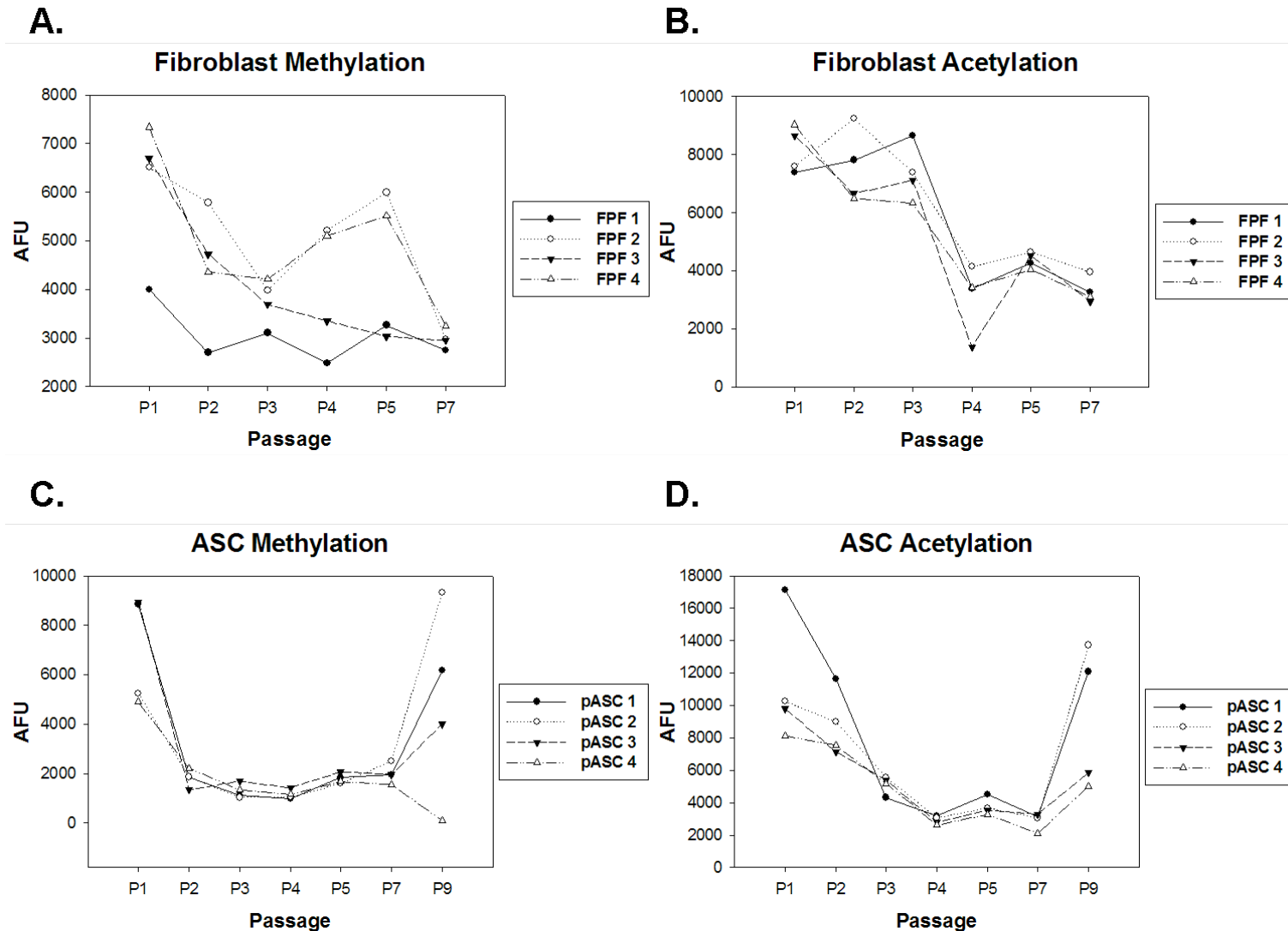


Figure 5.3. Global levels of methylated DNA and acetylated histone H3 in FPFs and pASCs at different passages. Cells gated in G0/G1 cell cycle stage based on PI counterstaining were designated a software-generated mean value. Each value was graphed for each of four cell lines and analyzed for FPF DNA methylation (A), FPF histone H3 acetylation (B), pASC DNA methylation (C) and pASC histone H3 acetylation (D).

Levels of Methylated DNA and Acetylated Histones in Porcine ASCs

Methylated DNA levels significantly decreased within the first passage for fibroblast cell lines FPF 1, 2, 3 and 4 gated in G0/G1 cell cycle stage (Table 5.2; Figure 5.3 A). However, the four lines appeared to diverge after passage 3 with methylated DNA levels remaining low for cell lines FPF 1 and 3 and increasing for cell lines FPF 2 and 4 before a decrease to lower levels at passage 7 (Figure 5.3 A). Relative levels of DNA methylation for pASCs also decreased significantly between passage 1 and passage 3 and remained low through culture (Table 5.2; Figure 5.3 C). As an average, pASC methylation levels were significantly lower than FPF levels from passage 2 through passage 7 (Table 5.2).

Levels of acetylated histone H3 for cells gated in G0/G1 cell cycle stage for FPF cell lines did not demonstrate a similar pattern between all four lines (FPF 1, 2, 3 and 4) through culture but all together did show a significant decrease in mean AFUs after passage 3 (Table 5.3; Figure 5.3 B). pASCs were hyperacetylated at passages 1 through 3 when mean relative levels significantly dropped at passage 4 (Table 5.2; Figure 5.3 D). When compared to FPFs, mean levels of acetylated histone H3 were significantly lower for pASCs only at passage 3, where as at all other passages analyzed the means were similar.

Analysis of Chromatin Remodeling Proteins Transcript Levels

mRNA levels for chromatin remodeling proteins Dnmt1, Dnmt3a, HDAC1 and MeCP2 were analyzed through RT-PCR. A mean fold change in gene expression relative to the calibrator and normalized with Poly A was determined for all four genes in FPFs and pASCs across passages 1, 2, 3, 4, 5, 7 and 9. Dnmt1 expression in pASCs increases through passage 3 and then tapers off during passage 3 through passage 9 (Table 5.4; Figure 5.4 A). FPF expression levels of Dnmt1 do not appear to follow any specific pattern through culture. The nature of passage 1 Dnmt1 expression level data is difficult to interpret and from passage 2 on the levels oscillate up and down (Table 5.4; Figure 5.4 A). However, when comparing the pASC

Dnmt1 expression pattern through culture to that of FPF, the levels are generally lower (Table 5.4; Figure 5.4 A).

Dnmt3a expression in pASCs follows a similar pattern to the expression of Dnmt1. Through passage 3, Dnmt3a expression levels increase and then begin to decrease after passage 3 through passage 9 (Table 5.5; Figure 5.4 B). Again, FPF expression levels of Dnmt3a, similar to Dnmt1, do not follow a noted trend through culture (Table 5.5; Figure 5.4 B). The Dnmt3a pattern of the FPFs is less consistent than the pASC pattern and again the pASC levels are relatively lower than FPF levels (Table 5.5; Figure 5.4B). Also, for both cell types the expression level for Dnmt3a as a whole is lower than that of Dnmt1.

The transcript levels for HDAC1 remained generally low throughout culture for both pASCs and FPFs with the exception of passage 1 and 9 for FPFs (Table 5.6; Figure 5.4 C). MeCP2 expression levels in pASCs, as expected, follow a similar trend through *in vitro* culture as Dnmt1 and Dnmt3a. Transcripts levels increase through passage 3 and are followed by subsequent decrease through passage 9 (Table 5.7; Figure 5.4 D). The abundance of MeCP2, as a whole, is more comparable to that of Dnmt1 hovering around a 1-fold change relative to the calibrator, whereas Dnmt3a levels are all under a 0.5-fold change (Tables 5.4, 5.5, 5.7; Figure 5.4). FPF expression levels of MeCP2 again are less consistent and do not follow any sort of trend through culture (Table 5.7; Figure 5.4 D). pASC expression levels of MeCP2 are lower when compared to the expression levels in FPF (Table 5.7; Figure 5.4 D).

Chromatin Immunoprecipitation of H3K4me3 and H3K27me3 Promoter Regions

Bivalent domains within the promoter regions work to silence developmentally important transcription factors while simultaneously keeping them poised for activation. We analyzed pASCs for the presence of the repressive H3K27me3 epigenetic modification and the activating H3K4me3 epigenetic modification within the promoter region of Oct-4 and Nanog. Preliminary evidence suggests that pASCs do, in fact, possess this bivalent chromatin structure in the proximal promoter region of Oct-4 with a value of 27.97 ± 13.56 for H3K4me3, as derived as a

Table 5.4. Average Relative Transcript Levels of DNA methyltransferase 1 in FPFs and pASCs through *In Vitro* Culture

Cell Line	Fold Change in Dnmt1 Expression \pm SEM						
	P1	P2	P3	P4	P5	P7	P9
FPF 1	39.79 \pm 31.80	0.30 \pm 0.05	1.83 \pm 0.24	1.03 \pm 0.13	1.76 \pm 0.35	3.02 \pm 0.28	0.80 \pm 0.08
FPF 2	0.66 \pm 0.45	0.18 \pm 0.02	1.93 \pm 0.58	1.30 \pm 0.37	2.63 \pm 0.90	1.26 \pm 0.35	0.00 \pm 0.00
FPF 3	16.17 \pm 11.46	0.20 \pm 0.03	4.08 \pm 1.39	0.87 \pm 0.11	1.22 \pm 0.09	1.30 \pm 0.27	0.70 \pm 0.08
FPF 4	0.41 \pm 0.07	0.12 \pm 0.01	1.07 \pm 0.13	1.12 \pm 0.12	1.08 \pm 0.03	1.17 \pm 0.04	0.57 \pm 0.07
Mean	14.26 \pm 9.28 ^{1,a}	0.20 \pm 0.04 ^{2,a}	2.23 \pm 0.65 ^{2,a}	1.08 \pm 0.09 ^{2,a}	1.67 \pm 0.35 ^{2,a}	1.69 \pm 0.45 ^{2,a}	0.52 \pm 0.18 ²
pASC 1	0.34 \pm 0.05	0.23 \pm 0.03	2.85 \pm 0.38	0.46 \pm 0.03	0.33 \pm 0.04	0.36 \pm 0.03	0.28 \pm 0.01
pASC 2	0.39 \pm 0.05	0.30 \pm 0.03	1.42 \pm 0.22	0.69 \pm 0.05	0.65 \pm 0.09	0.72 \pm 0.04	0.32 \pm 0.01
pASC 3	0.39 \pm 0.06	0.56 \pm 0.07	0.33 \pm 0.04	0.84 \pm 0.06	0.91 \pm 0.15	0.43 \pm 0.05	0.23 \pm 0.01
pASC 4	0.20 \pm 0.03	0.36 \pm 0.02	1.87 \pm 0.41	0.52 \pm 0.52	0.37 \pm 0.04	0.36 \pm 0.05	0.24 \pm 0.04
Mean	0.33 \pm 0.04 ^{1,a}	0.36 \pm 0.07 ^{1,a}	1.62 \pm 0.52 ^{1,a}	0.63 \pm 0.09 ^{1,b}	0.57 \pm 0.14 ^{1,b}	0.47 \pm 0.09 ^{1,b}	0.27 \pm 0.02 ¹

SEM, standard error of the mean.

Different letters (a and b) within columns indicate significant difference between cell types.

Different numbers (1 and 2) within rows indicate significant difference between passages.

ANOVA with repeated measures utilizing the Proc Mixed Procedure was used to determine statistical differences ($P < 0.05$).

Table 5.5. Average Relative Transcript Levels of DNA methyltransferase 3a in FPFs and pASCs through *In Vitro* Culture

Cell Line	Fold Change in Dnmt3a Expression \pm SEM						
	P1	P2	P3	P4	P5	P7	P9
FPF 1	2.50 \pm 2.15	0.17 \pm 0.06	0.32 \pm 0.03	0.25 \pm 0.08	0.31 \pm 0.15	1.73 \pm 0.58	0.07 \pm 0.01
FPF 2	0.03 \pm 0.01	0.08 \pm 0.03	0.25 \pm 0.04	0.29 \pm 0.02	0.30 \pm 0.02	0.33 \pm 0.10	0.00 \pm 0.00
FPF 3	0.66 \pm 0.62	0.19 \pm 0.09	0.47 \pm 0.07	0.16 \pm 0.06	0.34 \pm 0.01	0.19 \pm 0.05	0.70 \pm 0.05
FPF 4	0.27 \pm 0.01	0.12 \pm 0.08	0.12 \pm 0.01	0.15 \pm 0.03	0.30 \pm 0.02	0.17 \pm 0.03	0.69 \pm 0.02
Mean	0.86 \pm 0.56 ^{1,a}	0.14 \pm 0.03 ^{2,a}	0.29 \pm 0.07 ^{2,a}	0.21 \pm 0.03 ^{2,a}	0.31 \pm 0.01 ^{2,a}	0.60 \pm 0.38 ^{1,a}	0.36 \pm 0.19 ^{1,a}
pASC 1	0.09 \pm 0.02	0.04 \pm 0.02	0.54 \pm 0.37	0.03 \pm 0.03	0.02 \pm 0.01	0.01 \pm 0.00	0.04 \pm 0.02
pASC 2	0.07 \pm 0.01	0.08 \pm 0.01	0.21 \pm 0.04	0.07 \pm 0.01	0.08 \pm 0.01	0.14 \pm 0.02	0.07 \pm 0.02
pASC 3	0.07 \pm 0.02	0.14 \pm 0.03	0.11 \pm 0.01	0.20 \pm 0.02	0.12 \pm 0.02	0.06 \pm 0.02	0.04 \pm 0.01
pASC 4	0.04 \pm 0.01	0.04 \pm 0.02	0.41 \pm 0.41	0.11 \pm 0.03	0.06 \pm 0.01	0.05 \pm 0.01	0.05 \pm 0.01
Mean	0.07 \pm 0.01 ^{1,a}	0.07 \pm 0.02 ^{1,a}	0.32 \pm 0.10 ^{1,a}	0.10 \pm 0.04 ^{1,a}	0.07 \pm 0.02 ^{1,b}	0.06 \pm 0.03 ^{1,a}	0.05 \pm 0.01 ^{1,a}

SEM, standard error of the mean.

Different letters (a and b) within columns indicate significant difference between cell types.

Different numbers (1 and 2) within rows indicate significant difference between passages.

ANOVA with repeated measures utilizing the Proc Mixed Procedure was used to determine statistical differences ($P < 0.05$).

Table 5.6. Average Relative Transcript Levels of Histone Deacetylase 1 in FPFs and pASCs through *In Vitro* Culture

Cell Line	Fold Change in HDAC1 Expression \pm SEM						
	P1	P2	P3	P4	P5	P7	P9
FPF 1	13.93 \pm 13.15	0.12 \pm 0.01	0.28 \pm 0.04	0.19 \pm 0.03	0.26 \pm 0.05	0.54 \pm 0.05	0.11 \pm 0.00
FPF 2	0.02 \pm 0.02	0.15 \pm 0.03	0.35 \pm 0.05	0.23 \pm 0.05	0.52 \pm 0.19	0.24 \pm 0.06	1.44 \pm 1.18
FPF 3	0.14 \pm 0.14	0.13 \pm 0.03	1.07 \pm 0.37	0.17 \pm 0.01	0.25 \pm 0.01	0.23 \pm 0.03	0.78 \pm 0.08
FPF 4	0.12 \pm 0.02	0.06 \pm 0.00	0.20 \pm 0.01	0.26 \pm 0.02	0.18 \pm 0.01	0.25 \pm 0.02	0.85 \pm 0.09
Mean	3.55 \pm 3.46 ^{1,a}	0.11 \pm 0.02 ^{2,a}	0.48 \pm 0.20 ^{2,a}	0.21 \pm 0.02 ^{2,a}	0.30 \pm 0.07 ^{2,a}	0.32 \pm 0.08 ^{2,a}	0.80 \pm 0.27 ^{2,a}
pASC 1	0.16 \pm 0.02	0.12 \pm 0.01	0.53 \pm 0.09	0.15 \pm 0.01	0.08 \pm 0.01	0.13 \pm 0.01	0.13 \pm 0.00
pASC 2	0.18 \pm 0.03	0.13 \pm 0.01	0.35 \pm 0.07	0.17 \pm 0.01	0.11 \pm 0.01	0.35 \pm 0.02	0.14 \pm 0.01
pASC 3	0.19 \pm 0.01	0.21 \pm 0.01	0.14 \pm 0.01	0.23 \pm 0.01	0.16 \pm 0.02	0.13 \pm 0.01	0.11 \pm 0.00
pASC 4	0.11 \pm 0.01	0.32 \pm 0.01	0.27 \pm 0.11	0.14 \pm 0.03	0.10 \pm 0.01	0.14 \pm 0.02	0.08 \pm 0.01
Mean	0.16 \pm 0.02 ^{1,a}	0.20 \pm 0.04 ^{1,a}	0.32 \pm 0.08 ^{1,a}	0.17 \pm 0.02 ^{1,a}	0.11 \pm 0.02 ^{1,b}	0.19 \pm 0.05 ^{1,a}	0.12 \pm 0.01 ^{1,b}

SEM, standard error of the mean.

Different letters (a and b) within columns indicate significant difference between cell types.

Different numbers (1 and 2) within rows indicate significant difference between passages.

ANOVA with repeated measures utilizing the Proc Mixed Procedure was used to determine statistical differences ($P < 0.05$).

Table 5.7. Average Relative Transcript Levels of Methyl CpG-Binding Protein-2 in FPFs and pASCs through *In Vitro* Culture

Cell Line	Fold Change in MeCP2 Expression \pm SEM						
	P1	P2	P3	P4	P5	P7	P9
FPF 1	68.95 \pm 33.65	0.75 \pm 0.12	1.89 \pm 0.15	1.28 \pm 0.07	2.08 \pm 0.20	8.03 \pm 1.73	0.98 \pm 0.08
FPF 2	2.32 \pm 0.75	0.38 \pm 0.05	2.30 \pm 0.32	1.43 \pm 0.30	2.41 \pm 0.87	3.58 \pm 1.00	260.66 \pm 169.19
FPF 3	283.70 \pm 279.83	0.46 \pm 0.08	5.90 \pm 2.03	1.11 \pm 0.11	1.16 \pm 0.08	2.39 \pm 0.13	4.29 \pm 0.48
FPF 4	0.33 \pm 0.05	0.15 \pm 0.01	1.12 \pm 0.11	1.24 \pm 0.09	1.02 \pm 0.13	2.11 \pm 0.15	7.02 \pm 0.37
Mean	88.83 \pm 66.89 ^{1,a}	0.44 \pm 0.12 ^{2,a}	2.80 \pm 1.06 ^{2,a}	1.26 \pm 0.07 ^{2,a}	1.67 \pm 0.34 ^{2,a}	4.03 \pm 1.37 ^{2,a}	68.24 \pm 64.15 ^{1,a}
pASC 1	0.48 \pm 0.08	0.57 \pm 0.04	3.55 \pm 0.69	0.53 \pm 0.09	0.25 \pm 0.02	0.36 \pm 0.01	0.54 \pm 0.08
pASC 2	0.46 \pm 0.06	0.50 \pm 0.03	1.58 \pm 0.30	0.82 \pm 0.03	0.40 \pm 0.01	0.96 \pm 0.32	0.32 \pm 0.02
pASC 3	0.46 \pm 0.12	0.68 \pm 0.05	0.35 \pm 0.01	0.95 \pm 0.11	0.50 \pm 0.07	0.29 \pm 0.03	0.24 \pm 0.03
pASC 4	0.30 \pm 0.10	0.62 \pm 0.05	1.27 \pm 0.84	0.86 \pm 0.21	0.29 \pm 0.04	0.33 \pm 0.07	0.27 \pm 0.02
Mean	0.43 \pm 0.04 ^{1,a}	0.59 \pm 0.04 ^{1,a}	1.69 \pm 0.67 ^{1,a}	0.79 \pm 0.09 ^{1,b}	0.36 \pm 0.06 ^{1,b}	0.49 \pm 0.16 ^{1,a}	0.34 \pm 0.07 ^{1,a}

SEM, standard error of the mean.

Different letters (a and b) within columns indicate significant difference between cell types.

Different numbers (1 and 2) within rows indicate significant difference between passages.

ANOVA with repeated measures utilizing the Proc Mixed Procedure was used to determine statistical differences ($P < 0.05$).

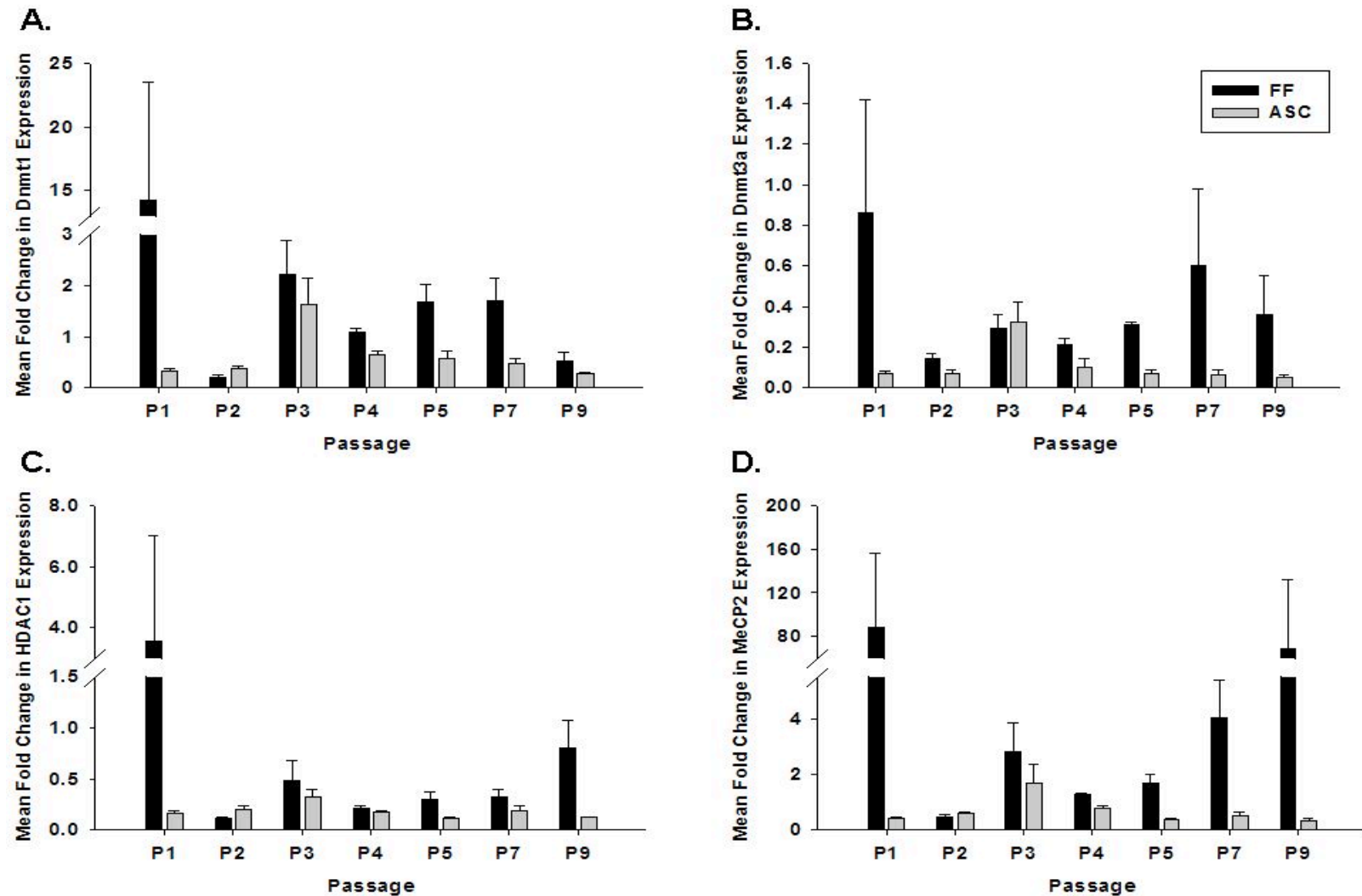


Figure 5.4. Histograms of the mean transcript levels in FPFs and pASCs for DNA methyltransferase-1 (Dnmt1), DNA methyltransferase-3a (Dnmt3a), histone deacetylase-1 (HDAC1) and methyl CpG binding protein-2 (MeCP2). Means are reported as a fold change in expression of each gene relative to a calibrator from porcine muscle tissue. Relative differences are depicted for both FPFs and pASCs for expression of Dnmt1 (A), Dnmt3a (B), HDAC1 (C) and MeCP2 (D). Bars are representative of the standard error of the mean.

Table 5.8 Chromatin Immunoprecipitation for Bivalent Domain Epigenetic Modifications

Gene Promoter	Percent + SEM	
	Lysine 4	Lysine 27
pOCT4 Proximal	27.97 \pm 13.56	31.81 \pm 6.13
pOCT4 Distal	3.21 \pm 0.99	7.34 \pm 2.01
pNanog Proximal	1.40 \pm 0.77	0.86 \pm 0.84
pNanog Distal	0.26 \pm 0.18	0.06 \pm 0.03

p = Porcine.

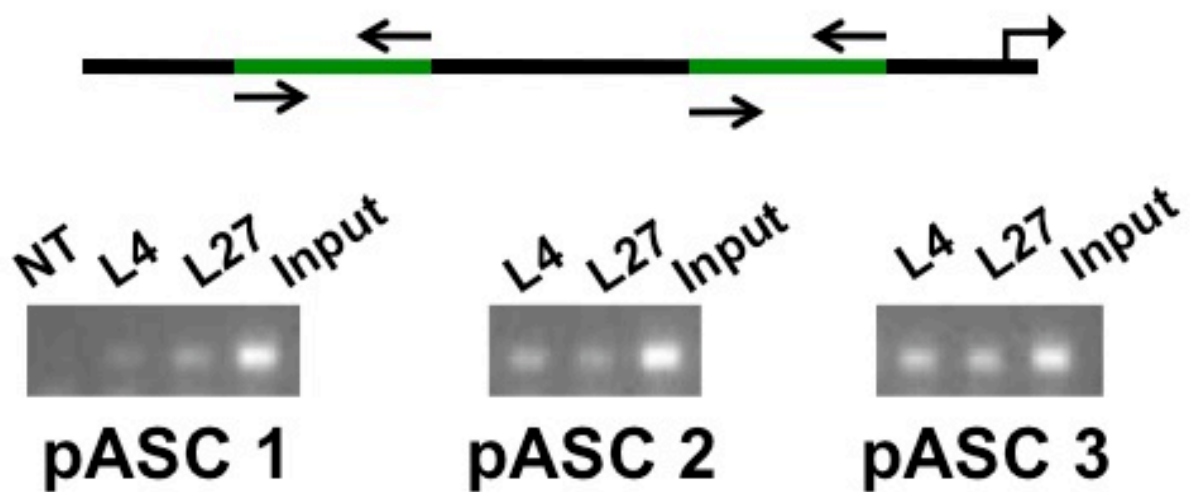


Figure 5.5 Proximal and Distal Promoter Regions of Oct-4 and Nanog. Primers were designed for regions proximal and distal to the initiation of transcription (shown in green). ChIP procedure was performed for immunoprecipitated chromatin containing the H3K4me3 and H3K27me3 modifications and real-time PCR was performed to determine the presence of the modifications within the promoter regions of Oct-4 and Nanog. Transcripts were run on an electrophoresis gel for visualization.

proportion of the input chromatin, and 31.81 ± 6.13 for the H3K27me3 epigenetic mark. The Oct-4 distal promoter region generated lower values of 3.21 ± 0.99 for lysine 4 and 7.34 ± 2.01 for lysine 27. Nanog proximal (1.40 ± 0.77 and 0.86 ± 0.84) and distal (0.26 ± 0.18 and 0.06 ± 0.03) promoters generated low values for each modification lysine 4 and lysine 27, respectively.

Discussion

Over a decade after the successful production of the first viable cloned sheep offspring named Dolly (Wilmut et al., 1997), the efficiency of NT remains remarkably low resulting in approximately 1 to 2% live births (Hiendleder et al., 2005). Research initially focused on factors surrounding crucial synchronization of the cell cycle phase of the donor nucleus and the recipient cytoplasm, but has shifted to investigate the mechanisms involved in adequate reprogramming of the donor genome.

During normal mammalian development, periods of genome-wide reprogramming of epigenetic patterns are believed to occur. The events following fertilization of the oocyte generate many different types of cells that make up the developing organism. A distinguishing pattern of epigenetic mechanisms summons differential subsets of gene activities without altering the DNA sequence. Resultant epigenetic modifications, including histone modifications and DNA methylation, direct the cells produced from the fertilized oocyte to differentiate into a specialized cell type or tissue. The sophisticated genetic coordination regulates proper temporal and spatial expression of these genes throughout development (Lieb et al., 2006; Shiota, 2004).

The procession of these events must be preceded by the removal of acquired epigenetic modifications instated by genetic predisposition or environment in germ cells for normal development and donor cells for NT (Allen et al., 1990; Engler et al., 1991; Reik et al., 1993; Sapienza et al., 1989). The failure of NT embryos to develop normally may be attributed to inherited epigenetic information from the somatic donor cell by the embryo as a result of incomplete erasure of the donor genome (Santos and Dean, 2004). Therefore, the use of a cell

such as a stem cell with low or absent levels of chromatin remodeling proteins such as Dnmt1, Dnmt3a, HDAC1 and MeCP2 may correlate to low levels of DNA methylation translating into more complete reprogramming in NT embryos. In this study, the gene expression profiles of epigenetic factors and their resultant modifications in differentiated fetal porcine fibroblasts (FPF) and undifferentiated porcine adipose tissue-derived stem cells (pASCs) at multiple passages through *in vitro* culture.

The current techniques employed today to establish and maintain primary *in vitro* cell cultures may be responsible for the induction of cytokinetic abnormalities and chromosomal alterations (Chu, 1962; Denning et al., 2001; Kasinathan et al., 2001; Oback and Wells, 2002; Rubin, 1997). We observed marked changes in the levels of mRNA for the chromatin remodeling proteins analyzed through culture in both FPFs and pASCs. However, the variation was much more substantial in the FPF cell lines when compared to the pASC cell lines. None of the cell lines analyzed were frozen at any point in the study; therefore neither the effects of cryoprotectants nor the freezing procedure itself can be responsible for the observed changes in transcript expression. However, the cellular stresses induced during the initial establishment of the cell cultures especially in FPFs most likely attributed to the variation of mRNA expression levels as well as the high levels of both DNA methylation and histone acetylation in the analysis of passage 1 cell lines. Spontaneous transformations reported to accumulate proportionally to the time in culture (Oback and Wells, 2002) may also contribute to the noted changes over time in *in vitro* culture.

Dnmt1 directly interacts with MeCP2 to recognize hemi-methylated DNA while HDAC1 works with both Dnmt1 and MeCP2 to deacetylate histones (Nan et al., 1998). As a unit these proteins function to maintain DNA methylation over multiple generations in culture (Kimura and Shiota, 2003) forming a synergism between histone acetylation and DNA methylation. In addition, Dnmt3a will also function as a maintenance enzyme since a knockout demonstrates a decreased level of DNA methylation over time in culture (Chen et al., 2003). Therefore, DNA

methylation would be expected to increase in response to an increase in the prevalence of these three proteins.

In this study, the messenger RNA pattern detected in pASCs for Dnmt1, Dnmt3a, HDAC1 and MeCP2 all followed the same pattern through culture with a noted increase in expression at passage 3 followed by a subsequent decrease through passage 9. A possible explanation of the increase in the expression levels of these proteins at passage 3 may be that during the first two passages the cells are battling the initial stress of establishing the culture and that by passage 3 the cells have reinstated normal cellular processes. Nevertheless, expression levels of these proteins through *in vitro* culture remain low and correlate to the expected low level of methylated DNA during passages 2 through 7. Interestingly, the low level of DNA methylation for pASCs remained consistently low over passages 2 through 7, which was not observed to be true for FPF cell lines similar to those currently employed for NT. This finding suggests that pASCs may serve as more efficient donor cells for NT because a lower level of DNA methylation may be more easily reprogrammed following fusion with the enucleated oocyte.

The expression levels of HDAC1 in pASCs and FPFs were also observed at relatively low levels throughout culture. The low abundance parallels the lower levels found for the other three chromatin remodeling proteins. In relation to the expression levels of the HDAC1 transcript, histone H3 acetylation patterns in pASCs were relatively low from passage 3 through passage 7. This trend roughly correlates to the observed low levels of DNA methylation at passages 2 through 7.

Primary cells utilized to establish culture possess the capacity to survive only a finite number of population doublings before cellular senescence is reached (Hayflick and Moorhead, 1961). As cells approach this limit, the rate of proliferation decreases (Cameron, 1972) and the probability of cells to undergo cytokinetic abnormalities and chromosomal alterations increases substantially (Giraldo et al., 2006; Oback and Wells, 2002). We observed an altered display of

DNA methylation and histone H3 acetylation in the initial cultures as well as the late passage 9 in pASCs. The variation at these time points in culture may be attributed to the establishment of the cell culture *in vitro* for the first few passages and the accumulation of chromosomal abnormalities for the late passage 9 cell populations. FPF cell lines also exhibited a high degree of variation in DNA methylation and histone H3 acetylation through culture. These findings are consistent with the pattern of epigenetic modifications detected in bovine fetal fibroblasts (Giraldo et al., 2007). The presence of a heterogeneous cell population, a greater susceptibility to spontaneous transformation or characteristics exclusive to the particular cell line may be potential culprits of the variation.

The inconsistent patterns of DNA methylation and histone acetylation found in FPF cell lines are probably more likely than pASCs to induce higher methylation levels found in cloned embryos. A study using bovine fibroblasts showed that although bovine fibroblast nuclei initially lost some methylation following fusion with the oocyte, further demethylation was not observed and instead *de novo* methyltransferases prematurely reinstated levels back to resemble that in differentiated nuclei (Dean et al., 2001). Somatic cell-like methylation patterns found in cloned embryos such as these may be a result of the introduction of the somatic version of Dnmt1 upon fusion with the recipient oocyte, which functions to maintain methylation patterns characteristic to the cell type in which it was derived (Reik et al., 2001). Thus a requirement for successful NT is the sufficient erasure of inherited epigenetic information from the differentiated donor cell nuclei.

In conclusion, *in vitro* cultured cells demonstrated altered mRNA transcript levels of chromatin remodeling proteins, which were consistently lower in pASCs than in FPFs. Also, FPF cell lines displayed greater variability in DNA methylation and histone H3 acetylation patterns than pASCs. We suggest that cultured pASCs, which display a consistently lower DNA methylation pattern as we described, may be more conducive to proper reprogramming necessary for improved efficiency of NT. Also, preliminary evidence suggests the presence of a

stem cell-associated bivalent chromatin structure harboring both H3K4me3 and H3K27me3 epigenetic modifications within the promoter region of Oct-4, providing further evidence that these cells possess this stem cell characteristic. Subsequent employment of these cells in NT procedures will elucidate the true efficacy of pASCs as donor cells for NT.

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CHAPTER VI

SUMMARY AND CONCLUSIONS

Although considerable amounts of research have focused on the intricacies involved in the process of NT, the efficiency of the procedure remains low with a 1 to 2% success rate. In addition to the low efficiency, anomalies resulting from suboptimal NT ultimately jeopardize the health and welfare of any produced cloned offspring generating problems such as those symptoms associated with Large Offspring Syndrome. Research has previously focused on factors such as the improper synchrony of the cell cycle phase of the donor cell nucleus with the recipient ooplasm and more recently has shifted to investigating adequate reprogramming of the donor genome.

The failure of NT embryos to develop normally may be attributed to inherited epigenetic information from the somatic donor cell by the embryo as a result of incomplete erasure of the donor genome. Therefore, the use of a cell such as a stem cell with low or absent levels of the enzymes responsible for creating the epigenetic landscape such as Dnmt1, Dnmt3a, HDAC1 and MeCP2 may correlate to a lower incidence of inherited epigenetic marks carried over from the cell type in which the donor cell was derived. The incorporation of a donor cell with low levels of DNA methylation initially may ultimately translate into more complete reprogramming in NT embryos.

Research in stem cell biology has exploded over the last two decades. However, the basic biology of *in vitro* culture continues to be elucidated. The improvement of culture conditions and the definition of cellular characteristics will aid the efficient isolation and purification of stem cell populations from multiple species-specific tissues creating important animal models for biomedical applications. Specific to this project, current knowledge of the optimal culture conditions for pluripotent and differentiated MSCs from adipose tissue, an easily attainable tissue source have not been defined. Also, culture characteristics of these cells have

not been clearly defined and studies generating gene and protein expression profiles are underway, but remain inconclusive.

Stem cell characteristics must be clearly defined in order to isolate, identify and optimally culture potential donor cells for NT. Stem cell characteristics such as self-renewal and differentiation have not been identified until now in stem cells from porcine adipose tissue. In addition, the phenotypic profile of cell surface proteins and chromosomal stability of porcine adipose tissue-derived stem cells (pASCs) through *in vitro* culture has not been described. At this time, research has not identified epigenetic modifications in adult somatic MSCs from porcine adipose tissue.

In Project 1 we developed a successful protocol for the isolation and culture of pASCs and determined stem cell-like characteristics. Initial culture of pASCs generated similar quantities of cells to those of other species-specific fat depots reported in the literature. pASC cell cultures preferred a higher seeding density in order for rapid colony cultivation to ensue. The duration of culture pASCs can undergo without reaching replicative senescence was comparable to those published for human adipose tissue and porcine bone marrow aspirates and the cell cycle lengths although a little longer for pASCs were also very similar to other species-specific tissues.

The cloning capacity of pASCs was determined using a ring cloning procedure. Multiple colonies were identified establishing the ability of pASCs to self-renew. The colonies were then subjected to differentiation conditions and found to differentiate successfully into osteogenic, adipogenic and chondrogenic tissue lineages.

Continuing our investigation of the basic biology of pASCs in Project 2, we described the cell surface profile for stem cell associated surface markers comparing early and late passages of undifferentiated porcine ASCs. The surface phenotype of porcine ASCs is similar for some stem cell antigens and different for others when compared with human ASCs cell surface, as reported in the literature. Different from reported values for human ASCs, porcine ASCs did not

demonstrate positive expression for CD44 or SSEA-4. We confirmed the absence of CD44 by western blot analysis. It is important to note that Project 1 was extracted from our publication that included a study, which did, in fact, reveal positive gene expression of CD44 through reverse transcriptase polymerase chain reaction. SSEA-4, found on bone marrow MSCs and human ASCs, and Sca-1, also found on MSCs, were not detected on the surface of porcine ASCs at early or late PDs. We did find consistent with the literature, however, CD29 and CD90 expression detected in early and late PDs while CD8 and CD117 antigens were not present at early or late PDs. Our study is by no means a comprehensive investigation for stem cell markers potentially present on the surface of porcine ASCs and many others will need to be analyzed as antibodies become available.

The chromosomal stability of pASCs through *in vitro* culture was found to be similar to bovine fetal fibroblasts as published in the literature. pASCs demonstrated an increased percentage of aneuploid cells directly from the initiation of culture with almost a 50% decrease in the incidence of normal diploid cells. The percentage of aneuploid cells significantly increased for middle PDs and late PDs. The assault to chromosomal stability clearly occurs after removing the cells from their *in vivo* environment, which is supported by such elevated levels of aneuploidy directly from the start in both bovine fetal fibroblasts and porcine ASCs. The sub-optimal culture conditions continue to corrode the genetic stability of the cells as they fight to survive *in vitro* culture through middle and late PDs as indicated by the significant increases in aneuploidy.

In Project 3, we analyzed the gene expression profiles of epigenetic factors and their resultant modifications, DNA methylation and histone H3 acetylation, in differentiated FPF and undifferentiated pASCs at multiple passages through *in vitro* culture. We observed marked changes in the levels of mRNA for the chromatin remodeling proteins analyzed through culture in both FPFs and pASCs. However, the variation was much more substantial in the FPF cell lines when compared with the pASC cell lines. The messenger RNA pattern detected in pASCs

for Dnmt1, Dnmt3a, HDAC1 and MeCP2 all followed the same pattern through culture and remained low, which correlates with the expected low level of methylated DNA observed during passages 2 through 7. Interestingly, the observed low level of DNA methylation for pASCs remained consistently low over passages 2 through 7, which was not true for FPF cell lines similar to those currently employed for NT. In relation to the expression levels of HDAC1, histone H3 acetylation patterns in pASCs were relatively low from passage 3 through passage 7. This trend roughly correlates to the observed low levels of DNA methylation at passages 2 through 7. Also, we observed an altered display of DNA methylation and histone H3 acetylation in the initial cultures as well as the late passage 9 in pASCs. FPF cell lines also exhibited a high degree of variation in DNA methylation and histone H3 acetylation through culture.

We consider the possibilities of cellular stresses induced during the initial establishment of the cell cultures especially in FPFs and spontaneous transformations reported to accumulate proportionally to the time in culture as culprits to the variation of mRNA expression levels as well as the high levels of both DNA methylation and histone acetylation in the analysis of passage 1 cell lines. Also, the variation at initial culture and later in passage may be attributed to the initial events necessary to establishment cell culture and the accumulation of chromosomal abnormalities in later passages. The presence of a heterogeneous cell population, a greater susceptibility to spontaneous transformation or unique characteristics to the cell line under investigation may also be attributed with the variation noted in our results.

In conclusion, improved culture conditions and defined cellular characteristics of self-renewal of these pASCs have been identified. pASCs can self-renew and differentiate into multiple tissue lineages. The phenotypic landscape of pASCs is similar for some cell surface markers reported in human ASCs and different for others. *In vitro* cultured porcine ASCs used as donor cells for NT should be chosen from early PDs due to increased levels of aneuploidy at later PDs. pASCs may serve as more efficient donor cells for NT because a lower level of DNA methylation may be more easily reprogrammed following fusion with the enucleated oocyte. It is

suggested that cultured pASCs which display a consistently lower DNA methylation pattern as we described may be more conducive to proper reprogramming necessary for improved efficiency of NT. Subsequent employment of these cells in NT procedures will elucidate the true efficacy of pASCs as donor cells for NT.

APPENDIX A: PROTOCOLS

WESTERN BLOTTING PROTOCOL

1. Place 2-10% gels in running buffer.
2. Load Gels:
 - a. Load using 1:1 ratio of loading buffer:sample
 - b. 10 μ l MW stds –heat in water bath
 - c. Heat samples at 95°C for 5-10 minutes
3. Run gels at 100 V until standards reach the bottom of the gel (~1 ½ hour-3 hours).
4. Wash gel for coomassie stain in MQ Water 3 X for 5 minutes.
5. Stain gel in Coomassie Blue (Bio-Rad Catalog # 161-0786).
6. Equilibrate gel by transferring into cold transfer (Towbin's) buffer (\geq 15 min).
7. Prepare membrane 30 seconds in 100% Methanol.
8. Transfer membrane into cold Towbin's buffer 2-3 minutes.
9. Soak pads and filter paper in cold transfer buffer.
10. Prepare transfer sandwich (load on black side of gel holder cassette in order):
 - a. Pad
 - b. Filter paper
 - c. Gel
 - d. Membrane
 - e. Filter paper
 - f. Pad
11. Place red and black mini trans-blot module into gel tank with the black side in the center and banana plugs in middle.
12. Place the frozen ice block (frozen in -80°C) into gel tank.
13. Slide cassette into the red and black mini trans-blot module as follows:
 - a. Black side of the sandwich facing the black side of the mini trans-blot module (middle) and clear side facing the red (outside).
14. Fill tank with cold Towbin's buffer and a stir bar and place in ice bucket.
15. Place ice bucket on stir plate and pack ice around the transfer apparatus.
16. Run transfer at 90 volts for about 90 minutes (make sure bubbles appear between trans-blot module and ice block).
17. Remove cassette, disassemble, and place membrane in blocking buffer 2 to 3 hours (poke holes to denote where to cut membrane if probing with different antibodies).

18. Pour off blocking buffer and incubate with 1° Antibody according to predetermined dilution (1:2000) overnight at 4°C:
 - a. 20 ml of Blocking buffer
 - b. 10 µl primary antibody
19. Perform 5 X 10 minute washes in TBST.
20. Incubate with 2° Antibody according to predetermined dilution (1:3000) for 1 hour at room temperature:
 - a. 20 ml of Blocking buffer
 - b. 6.7 µl secondary antibody
21. Perform 5 X 10 minute washes in TBST.
22. Incubate in color reagents for 30 minutes or when color appears:
 - a. 1 x HRP = 20 ml
 - b. Bio-Rad Reagent A = 2 ml
 - c. Bio-Rad Reagent B = 120 µl

Alternatively,

23. a. Mix ECL reagents 1:1 and allow to warm to room temperature.
 - b. Blot off wash buffer onto a paper towel by capillary action.
 - c. Place membrane on a piece of saran wrap.
 - d. Apply ECL reagents to top of membrane allowing surface tension to hold volume.
 - e. Incubate for 1 minute.
 - f. Again blot off ECL reagents onto a paper towel by capillary action.
 - g. Place protein side down onto another piece of saran wrap and fold into a pocket.
 - h. Tape into film cassette and expose to xray film 2 minutes, 5 minutes, 20 to 30 minutes, overnight.
 - i. Develop film in dark room.

CHROMATIN IMMUNOPRECIPITATION PROTOCOL

DNA-protein Cross-linking, Cell Lysis and Chromatin Fragmentation:

1. Harvest cells by trypsinization per standard protocol.
2. Count cells and allocate 2.5×10^6 cells to ChIP.
3. Centrifuge cells 300 x g for 5 minutes and remove supernatant.
4. Resuspend cells in 515 μ l PBS (without calcium).
5. Add 34 μ l of 16% paraformaldehyde (gives 1% v/v) and vortex lightly.
6. Incubate at room temperature 8 minutes.
7. Add 62 μ l room temperature 10X glycine (final concentration 125 mM).
8. Incubate at room temperature 5 minutes.
9. Centrifuge cells 300 x g for 10 minutes at 4°C and remove supernatant.
10. Wash in 500 μ l PBS.
11. Centrifuge cells 300 x g for 10 minutes at 4°C and remove supernatant.
12. Repeat wash (steps 10 and 11) or alternatively flash freeze pellet in LN₂ and store at -80°C.
13. Resuspend in 500 μ l Lysis Buffer and vortex lightly.
14. Incubate on ice for 5 minutes.
15. Transport cells to sonicate.
16. Sonicate each sample on Branson Sonifier 250 Duty cycle 40% Level 4 for 10 x 20 pulses on ice with 1 minute pause in between each set of 20 pulses.
17. Centrifuge at 12,000 x g for 10 minutes at 4°C.
18. Remove supernatant and put in new tube.
19. Can store at -80°C or move on to next procedure.

Preparation of Antibody-Bead Complexes:

1. For each sample to be analyzed, remove 11 μ l well-suspended bead stock solution (Millipore MagnaChIP Kit, Catalog #MAGNA001).
2. Place single tube in magnet to capture beads and remove storage buffer.

3. Remove tube from magnet and resuspend beads in 500 μ l RIPA buffer.
4. Mix lightly and replace into magnet to capture beads and remove RIPA buffer.
5. Repeat wash (steps 2-4).
6. Resuspend beads in 10.5 μ l RIPA buffer for each sample (*i.e.*, 10.5 x # samples gives total volume to resuspend with).
7. Add 90 μ l RIPA buffer to 0.2 ml PCR tube (1 per sample).
8. Add 10 μ l pre-washed beads to each PCR tube and 2.4 μ g of antibody.
9. Incubate at 4°C for 2 hours at 40 rpm on VWR Hybridization Oven.

Immunoprecipitation:

1. Dilute chromatin to 3 ml with RIPA buffer supplemented with 30 μ l HALT protease inhibitor (approximately 100,000 cells in 100 μ l).
2. Make 100 μ l aliquots and store in -20°C.
3. Touch-spin PCR tubes containing antibody-bead complexes to remove contents from lid.
4. Place into ice-cold magnet to capture beads and remove RIPA buffer.
5. Remove tubes from magnet and resuspend in 100 μ l of aliquoted chromatin.
6. Incubate at 4°C overnight at 40 rpm on VWR Hybridization Oven.
7. Touch-spin PCR tubes, place in ice-cold magnet and remove RIPA buffer.
8. Remove tubes from magnet and resuspend in 100 μ l RIPA buffer.
9. Incubate at 4°C for 4 minutes at 40 rpm on VWR Hybridization Oven.
10. Repeat wash 2 times (steps 7-9).
11. Touch-spin PCR tubes, place in ice-cold magnet and remove RIPA buffer.
12. Resuspend beads in 100 μ l TE buffer.
13. Incubate at 4°C for 4 minutes at 40 rpm on VWR Hybridization Oven.
14. Touch-spin PCR tubes and transfer contents to new PCR tube.
15. Place on magnet to capture beads and remove TE buffer.

DNA Elution, Cross-link reversal and Proteinase K Digestion:

1. Add 150 µl Elution buffer and add SDS to 1% and proteinase K to 50 µg/ml.
2. Add 200 µl Elution buffer to another 100 µl aliquot of diluted chromatin and add proteinase K to 50 µg/ml.
3. Incubate at 68°C for 2 hours at 40 rpm on VWR Hybridization Oven.
4. Touch-spin tubes and place on magnet to capture beads.
5. Collect supernatant and transfer to new 1.5 ml tube.
6. Add 150 µl Elution buffer/SDS/proteinase K and resuspend beads.
7. Incubate at 68°C for 5 minutes at 40 rpm on VWR Hybridization Oven.
8. Touch-spin tubes and place on magnet to capture beads.
9. Collect supernatant and add to 1.5 ml tube.

DNA Isolation:

1. Add 500 µl Bind Reagent A (Millipore Kit) to each sample and vortex.
2. Transfer sample/Bind Reagent A to Spin filter in collection tube.
3. Centrifuge for 30 seconds at 12,500 x g.
4. Remove spin filter from collection tube and save collection tube while discarding liquid.
5. Replace spin filter into collection tube and add 500 µl Wash Reagent B.
6. Centrifuge for 30 seconds at 12,500 x g.
7. Remove spin filter from collection tube and save collection tube while discarding liquid.
8. Replace spin filter into collection tube.
9. Centrifuge for 30 seconds at 12,500 x g.
10. Discard collection tube containing residual liquid.
11. Place spin filter into new collection tube.
12. Add 130 µl Elution Buffer C directly onto center of white spin filter membrane.
13. Centrifuge for 30 seconds at 12,500 x g.

14. Remove spin filter and discard.
15. Analyze purified DNA by qPCR or store at -20°C.

TRI REAGENT® RNA ISOLATION PROTOCOL

1. Turn on centrifuge and let cool to 4° C.
2. Prep phase lock tubes by spinning at 3500 x g for 10 minutes.
3. Pour off medium from flasks.
4. For T75 add 8 ml Trizol reagent to each T75 flask and pass cell lysate through pipette several times. Incubate at room temperature 5 min knock on counter top to dissociate.
5. Use the scrapper to remove cells and transfer lysate to phase lock tube.
6. Incubate at room temperature 5 minutes to permit the complete dissociation of nucleoprotein complexes.
7. Add 0.8 ml BCP in hood.
8. Cap tubes and shake vigorously by hand for 15 to 30 seconds.
9. Incubate tubes at room temperature for 30 minutes to 1 hour.
10. Centrifuge at 12,000 g for 15 minutes at 4° C.
11. Transfer aqueous supernatant into a fresh tube.
12. Precipitate RNA by mixing with 4 ml isopropyl alcohol (for T75). Shake well.
13. Hold at -20° C overnite for optimal recovery.
14. Centrifuge at 12,000 g for 20 minutes at 4° C putting number side up. RNA will form a gel-like or white pellet on the side and bottom of the tube.
15. Pour off supernatant away from pellet.
16. Respin pellet at 12,000 g for 2 minutes at 4° C.
17. Wash the RNA pellet in 75% EtOH (prepared in DECP water) using at least 1 ml of EtOH per 1 ml of Trizol reagent (8 ml for T75—make 40 ml for 4 samples).
18. Vortex tube until pellet is floating. Can use pipette tip to push off sides.
19. Centrifuge at 15,000 g for 10 minutes.
20. Transfer pellet in 1 ml ethanol (left from previous step) and respin for 15 minutes in 1.5 ml tube.
21. Carefully remove ethanol supernatant with a pipette followed by a disposable pipette then a thirsty stick.

22. Briefly dry the RNA pellet with top off at 37° C for about 7 minutes.
23. Dissolve the RNA pellet in DEPC water about 25 ul and incubate in 55 to 60° C waterbath about 10 minutes if needed.

KARYOTYPING PROTOCOL

1. Thaw or passage cells 24 hours prior to cell synchronization.
2. Add 0.05 µg/ml of KaryoMax® to directly to cell culture.
3. Incubate 30 minutes to 3 h at 37°C.
4. Remove media and put to side.
5. Harvest cells by trypsinization.
6. Add harvested cells to medium from step 4.
7. Centrifuge at 1200 x g for 5 minutes.
8. Remove supernatant and SLOWLY resuspend cells in 3 ml pre-warmed hypotonic solution in 0.5 ml increments.
9. Incubate in 37°C water bath 5 minutes.
10. Centrifuge at 1200 x g for 5 minutes.
11. Remove supernatant.
12. SLOWLY add fixative (3:1) in 0.5 ml increments.
13. Incubate in 4°C for 10 minutes.
14. Centrifuge at 1200 x g for 5 minutes.
15. Remove supernatant.
16. Resuspend in 0.5 ml fresh fixative.
17. Store in -20°C at least overnight and up to 2 months.
18. Hold clean slide at an angle and place 3 to 5 10 µl drops of cell suspension and allow drops to spread down slide.
19. Allow slides to dry at least 10 minutes.
20. Flood slide with 4 x 1 ml of 1:1 fixative.
21. Allow slides to dry at least 10 minutes.
22. Drop slides into Giemsa stain for 15 minutes.
23. Remove slides from stain and gently rinse with deionized water.

24. Allow slides to dry completely.

25. Visualize under 100 X oil immersion.

APPENDIX B: REAGENT FORMULATIONS

WESTERN RUNNING BUFFER

REAGENT	COMPANY	CATALOG NUMBER	AMOUNT
10X Tris/Glycine/SDS	BIO-RAD	161-0732	100 ml
MQ Water			900 ml

Prepare on day of use.

WESTERN LOADING BUFFER

REAGENT	COMPANY	CATALOG NUMBER	AMOUNT
Laemmli buffer	BIO-RAD	161-0737	950 µl
beta-mercaptoethanol	Sigma	M-7522	50 µl

Prepare on day of use.

WESTERN TOWBIN'S BUFFER

REAGENT	COMPANY	CATALOG NUMBER	AMOUNT
Trizma-Base	Sigma	T-1503	3.03 g (25 mM)
Glycine	Sigma	G-8898	14.4 g (192 M)
Methanol	Sigma	M-1775	200 ml (20 %)
MQ Water			Bring up to 1 L

Prepare on day of use.

WESTERN TBST BUFFER

REAGENT	COMPANY	CATALOG NUMBER	AMOUNT
10X TBS	BIO-RAD	170-6435	100 ml
10% Tween 20	BIO-RAD	161-0781	5 ml
MQ Water			Bring up to 1 L
Prepare on day of use.			

WESTERN BLOCKING BUFFER

REAGENT	COMPANY	CATALOG NUMBER	AMOUNT
Non-fat Dry Milk	BIO-RAD	170-6404	10 g
TBST			200 ml
Prepare on day of use.			

ChIP LYSIS BUFFER

REAGENT	COMPANY	CATALOG NUMBER	AMOUNT
Trizma-HCl	Fluka	93313	5 ml (50 mM)
EDTA	Sigma	E7889	2 ml (10 mM)
10% SDS	Gibco	15553-035	10 ml (1 %)
HALT Proteases Inhibitor	Thermo-Scientific	1860932	100 µl
PMSF	Sigma		5 µl (1 mM)
Store at 4°C several months.			

ChP RIPA BUFFER

REAGENT	COMPANY	CATALOG NUMBER	AMOUNT
Trizma-HCl	Fluka	93313	1 ml (10 mM)
EDTA	Sigma	E7889	200 µl (1 mM)
EGTA	Sigma	E3889	50 ml (0.5 mM)
Triton X-100	Sigma	X-100	1 ml
10% SDS	Gibco	15553-035	1 ml (0.1 %)
Sodium deoxycholate	Sigma	D6750	0.1 g
NaCl	Sigma	S5886	0.818 g
MQ Water			Bring to 100 ml

Store at 4°C several months.

ChIP TE BUFFER

REAGENT	COMPANY	CATALOG NUMBER	AMOUNT
Trizma-HCl	Fluka	93313	250 µl (10 mM)
EDTA	Sigma	E7889	500 µl (10 mM)

Store at room temperature several months.

ChIP Elution BUFFER

REAGENT	COMPANY	CATALOG NUMBER	AMOUNT
Trizma-HCl	Fluka	93313	500 µl (20 mM)
EDTA	Sigma	E7889	250 µl (5 mM)
NaCl	Sigma	S5886	0.073 g
MQ Water			Bring up to 25 ml

Store at room temperature several months.

DEPC WATER

REAGENT	COMPANY	CATALOG NUMBER	AMOUNT
DEPC	Sigma	D5758	100 µl
MQ Water			100 ml

Incubate at 37°C 1 hr, autoclave 15 min.
Store at room temperature several months.

HYPOTONIC SOLUTION

REAGENT	COMPANY	CATALOG NUMBER	AMOUNT
Sodium Citrate	Sigma	S4641	0.9 g
MQ Water			100 ml

Filter and store at room temperature indefinitely.

3:1 FIXATIVE SOLUTION

REAGENT	COMPANY	CATALOG NUMBER	AMOUNT
Methanol	Sigma	M1775	15 ml
Acetic Acid	Sigma	A6283	5 ml

Prepare fresh on day of use.

1:1 FIXATIVE SOLUTION

REAGENT	COMPANY	CATALOG NUMBER	AMOUNT
Methanol	Sigma	M1775	10 ml
Acetic Acid	Sigma	A6283	10 ml

Prepare fresh on day of use.

GIEMSA STAINING SOLUTION

REAGENT	COMPANY	CATALOG NUMBER	AMOUNT
Giemsa Stain	Sigma	GS-500	2 ml
MQ Water			28 ml

Prepare fresh on day of use.

APPENDIX C: LETTER OF PERMISSION

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Kellie Williams <kwill72@tigers.lsu.edu>

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2 messages

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Williams KJ, Picou AA, Kish SL, Giraldo AM, Godke RA, Bondioli KR. Isolation and characterization of porcine adipose tissue-derived adult stem cells. 188(3):251-258 (2008).

Any necessary documentation can be mailed or emailed to the following addresses:

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Sincerely,

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VITA

Kellie Jean Williams was born in September, 1980 in Watertown, Wisconsin, to Karrie Jean Keller and Ricky Gene Williams. Kellie has a younger brother, Shane Randel Williams, who is pursuing his master's degree in international business and economic relations at Oklahoma State University (OSU). Kellie graduated from Owasso High School in Owasso, Oklahoma in 1999. Subsequently, she attended OSU where she obtained a bachelor's degree in biochemistry and molecular biology in 2003. Following her undergraduate career, Kellie attended the University of Oklahoma Health Sciences Center (OUHSC) in Oklahoma City where she completed her master's degree in biochemistry and molecular biology. At OUHSC, her research was entitled "The Critical Elements of Oligosaccharide Acceptor Substrates for the *Pasteurella multocida* Hyaluronan Synthase" and was performed under the guidance of Dr. Paul DeAngelis. During her undergraduate and master's degree programs, Kellie participated in an internship with Dr. J. Kevin Thibodeaux at the Tulsa Center for Fertility and Women's Health at Hillcrest Medical Center and it is through Dr. Thibodeaux that she learned about the doctoral program in reproductive physiology at Louisiana State University (LSU) led by Dr. Robert A. Godke, Boyd Professor, and Dr. Kenneth R. Bondioli, Associate Professor, in the field of reproductive physiology. Kellie is candidate for the Doctor of Philosophy degree in reproductive physiology in the School of Animal Sciences at LSU under the guidance of Drs. Kenneth R. Bondioli and Robert A. Godke.